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Survival Following Acute Coronary Artery Ligation Subsequent to Irradiation of Canine Heart.* (24501)

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Attempts to increase blood supply to the myocardium of the heart with coronary artery disease have been many. The approaches, primarily surgical, have been designed to protect the heart from effects of acute coronary occlusion by promoting intercoronary and extracoronary communications. These methods have utilized foreign irritants, tissue grafts, and various anastomotic and implantation procedures involving systemic vessels and coronary arteries(1). In an attempt to attain the same protective benefits of myocardial revascularization, without the necessity of surgical intervention, the use of cardiac irradiation as a therapeutic measure was investigated. It is our premise that successive small doses of radiation will produce dilatation of existing myocardial capillaries and precapillary arterioles which, in the presence of myocardal ischemia and the natural compensatory ability of cardiac tissue to develop collaterals, will persist and increase in number. To determine the effects of radiation on the canine heart, the following study was performed.

Methods. Mongrel dogs in good health weighing 10-20 kg were used, and divided into Radiation and Control Groups. Each radiation dog received a chest x-ray to determine heart location and size. This field was then mapped on the dog's thorax on both left and right sides. Irradiation was performed by means of 3 treatments to the left chest field and 2 to the right chest field during a 2 to 2½ week period. Treatments were given with a parallel opposing pair of fields ranging from 80 to 120 cm^2. Average separation of fields

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TABLE I. Effects of Acute Coronary Ligation on Survival (If Defibrillation Not Attempted).

Group	Total No. of dogs	No. surviving ligation and alive 24 hr post ligation	% of dogs surviving
Control			
Group A	5	0	0
" B	18	1	5.55
Radiation			
Group 1300 R	15	7	46.66
" 2000 R	18	8	44.44

was 10 cm. 200 kilovolt x-rays, which after filtration had half value layer of 0.9 mm copper at focal skin distance of 50 cm, were utilized. Total dose of radiation to the heart in one group was 1300 Roentgens. The other group received total dose of 2000 Roentgens to the heart. Prior to radiation, each dog was anesthetized with Sodium Nembutal (25-30 mg/kg body weight) to insure immobility during treatment. Four to 6 days following last treatment in the Radiation Groups, ligation of the anterior descending branch of the left coronary artery was performed through a left-sided thoracotomy in the fifth intercostal space. An endotracheal tube connected to an automatic respirator, utilizing compressed air, was employed. The anterior descending coronary artery was ligated exactly at its origin from left main coronary artery with 2 OO silk sutures. Sterile procedure was maintained. The thoracic cavity was closed in layers and crystalline penicillin 600,000 units given intramuscularly for 3 days. In the 1300 Roentgen group there was no attempt to defibrillate the animal once ventricular fibrillation developed following placement of coronary ligature. In the 2000 Roentgen group all dogs which fibrillated following ligature were routinely treated with cardiac massage, oxygen, electrical counter-shock, 100 mg of Pronestyl, and .5 mg of Oubain given in stated order and at appropriate time. In none of the dogs were the ligatures removed. To keep conditions equivalent, the control group was also anesthetized 5 times in a 2 to 2½ week period and operated upon 4 to 6 days later. An attempt was also made to defibrillate one group of control dogs. Control Group A—5 dogs. No attempt at defibrillation was made. Control Group B—18 dogs. An attempt at defibrillation was made. Radiation Group 1300 R—15 dogs. No attempt at defibrillation was made. Radiation Group 2000 R—18 dogs. An attempt at defibrillation was made.

TABLE II. Effect of Attempts at Defibrillation in Dogs with Ventricular Fibrillation.

Group	No. dogs which fibrillated	No. dogs defibrillated and alive 24 hr post ligation	% of dogs defibrillated
Control Group B	17	5	29.41
Radiation Group 2000 R	8	7	87.50

Results. The effect of acute coronary ligation on all groups, as measured by survival of animal, assuming that no defibrillation was attempted, is presented in Table I. As this type of fibrillation is not spontaneously reversible, all dogs that fibrillated would have died.

TABLE III. A Comparison of Total Survivors following Acute Coronary Ligation.

Group	Total No. of dogs	Total No. survivors 24 hr post ligation	% surviving
Control			
Group A	5	0	.0
" B	18	6 (includes 5 defibrillated)	33.33
Radiation			
Group 1300 R	15	7	46.66
" 2000 R	18	15 (includes 7 defibrillated)	83.33

Table II presents results when an attempt was made to defibrillate those dogs with ventricular fibrillation in Control Group B and Radiation Group 2000 R without removal of the coronary ligature.

A comparison of total survivors (straight survivors plus defibrillated dogs) is presented in Table III.

Discussion. Analysis of the results significantly indicates that those dogs which received cardiac irradiation prior to acute coronary ligation were less prone to fatal fibrillation following coronary occlusion than the Control Group. Since the entire distribution of the anterior descending coronary artery is

occluded by the ligature applied, it is assumed that prevention of fibrillation occurs by back flow of blood into this area from adjacent coronary vessels. The high mortality in the control group is evidence that this flow is not adequate normally.

Stanton(2) postulates that better survivals, following experimental procedures designed to protect the heart, arise from increased fibrillation threshold which the performance of many of these procedures might induce. Since these dogs have had no operation prior to ligation, one concludes that radiation alone has produced sufficient vascularity to account for the observed differences in survival and increased fibrillation threshold.

Also of interest is the large percentage of radiated dogs which responded to defibrillation without removal of the ligature. Gregg (3) states that "experimentally, in dogs, it is rarely possible to revive a heart with an occluded coronary artery unless the occlusion is first removed and the ischemic area flooded with arterial blood." The experience with the control group tends to indicate that this situation is not as rare as described. However, the percentage difference in ability to defibrillate dogs between both groups and the ease with which defibrillation was performed in the radiated group is of great significance.

Summary. A method of creating increased myocardial vascularity by means of cardiac irradiation has been investigated. On the basis of mortality figures, there is a significant difference between control and irradiated dogs when acute complete ligation of the anterior descending branch of left coronary artery is performed. Ability to defibrillate radiated dogs successfully with the coronary ligature intact is strikingly evident.†

† Histological, physiological and electrocardiographic studies of the effects of cardiac irradiation, now in progress, will be reported.

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Effect of Dietary Cholic Acid on *in vivo* Cholesterol Metabolism.* (24502)

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The authors have presented evidence(1) which supported the view that effectiveness of dietary cholic acid in promoting accumulation of liver cholesterol in the rat, fed cholic acid and cholesterol simultaneously, is due to ability of bile acids to block cholesterol degradation. Furthermore, in the absence of dietary cholesterol, feeding cholic acid caused only a small accumulation of liver cholesterol, because of simultaneous and equal decreases in rates of liver cholesterol synthesis and mobilization. Inasmuch as in the rat, cholesterol is largely metabolized to bile acids before excretion, these effects could be of major importance in maintenance of cholesterol balances. However, several points must be investigated before this finding can be seen in its true perspective.

The present investigation concerns: 1. Effect of length of time of cholic acid-feeding period on rate of liver cholesterol synthesis; 2. Relationship of serum bile acid concentration to rate of cholesterol synthesis in liver; and 3. Effect of dietary cholic acid on kidney and intestine cholesterol synthesis.

Procedures. Forty-eight Sprague-Dawley female albino rats, weighing 250-280 g were divided into 2 groups. The control group was maintained on basal diet consisting of 97% Ground Rockland Rat Ration and 3% corn

* This investigation was supported, in part, by grant from Michigan Heart Assn.

TABLE I. Effect of Dietary Cholic Acid on Serum and Liver Total Cholesterol, and on Serum Bile Acid, as a Function of Time.

Exp. period (days)	Serum total cholesterol		Liver total cholesterol		Serum bile acid	
	Control	Cholic acid*	Control	Cholic acid*	Control	Cholic acid*
	mg %		mg/g†		mg %	
1	95.4	99.4	2.41	3.03	2.53	3.14
3	81.6	89.2	2.51	2.75	1.96	5.39
5	89.4	85.7	2.69	3.16	2.41	4.16
7	86.6	91.5	2.58	2.88	2.37	4.05
10	90.4	84.2	2.44	2.85	2.73	5.13
16	83.3	94.7	2.53	2.62	3.13	7.66
21	86.0	90.9	2.68	2.72	2.54	5.38
Overall avg	87.5 ± 4.65	90.8 ± 5.18	2.54 ± .11	2.86 ± .18	2.52 ± .36	4.99 ± 1.43
P	.22		<.01		<.01	

* 0.5% cholic acid. † Wet wt. ± numbers are stand. dev.

oil; the experimental group received basal diet supplemented with 0.5% cholic acid. both groups were fed *ad lib.*, average daily food consumption being 18.1 ± 1.46 g for controls and 17.0 ± 1.32 g for treated rats. After 1, 3, 5, 7, 10, or 16 days on their respective diets, 3 control and 3 cholic acid-fed rats were injected intraperitoneally with 50 microcurie of sodium acetate-1-C^{14} in saline. Six hours after injection, blood was drawn by heart puncture, after which the animals were sacrificed and liver samples removed for analysis. In addition, 6 control and 6 cholic acid-fed rats, which had been maintained on their respective diets for 21 days, were injected as previously described. Half of these animals were sacrificed 30 minutes and the other half 6 hours after injection. Blood was drawn by heart puncture, and after decapitation, samples of liver, kidney and small intestine were removed for analysis. Serum cholesterol was determined by the method of Sperry and Webb(2); serum bile acid by a combination of methods of Minibeck and Wilken(3,4). Liver, kidney and intestine cholesterol was determined by a method described previously (5). For determination of radioactivity, tissue cholesterol was isolated as the digitonide and counted(1).

Results. Table I presents levels of serum total cholesterol, liver total cholesterol and serum bile acids. Dietary cholic acid caused no significant change in serum cholesterol concentration during the experiment. However, a small but highly significant increase in liver total cholesterol occurred after one day of cholic acid feeding. This increase remained almost constant for the balance of the 21-day period. One day of cholic acid treatment resulted in a slight increase in serum bile acid level. There was a further increase after 3 days treatment to a level that remained relatively constant for the rest of the experiment.

The effect of dietary cholic acid on incorporation of acetate-1-C^{14} into liver and serum cholesterol is shown in Table II. Feeding cholic acid for one day had no effect on rate of liver cholesterol synthesis. After 3 days of cholic acid feeding, rate of synthesis was reduced by approximately 65%. This reduction remained at about the same level for the rest of the experiment. The same pattern was found in the case of appearance of cholesterol-x-C^{14} in blood.

It is of interest to note that serum bile acid concentration reached a maximum level after 3 days of cholic acid feeding (Table I). This same interval was necessary for reduction of

TABLE II. Incorporation of Acetate-l-C^{14} into Liver and Serum Total Cholesterol in Control Rats and Rats Fed 0.5% Cholic Acid for Different Time Intervals.

Exp. period (days)	Liver total cholesterol		Serum total cholesterol	
	Control	Cholic acid	Control	Cholic acid
	counts/min./mg			
1	2,828	2,798	2,316	2,311
3	3,238	1,075	3,023	1,120
5	2,981	1,362	2,384	1,304
7	3,111	980	2,745	829
10	3,901	1,008	3,220	923
16	3,406	1,064	3,610	966
21	3,798	1,168	3,219	893

liver cholesterol synthesis (Table II). On the other hand, after one day of dietary cholic acid, liver cholesterol was slightly but significantly elevated. These findings suggest that the decrease in liver cholesterol synthesis is more closely related to serum bile acid concentration than to the slight increase in liver cholesterol level. However, a more detailed study is necessary to decide this point. Inhibition of cholesterol synthesis by cholic acid could very well be a feed-back reaction. Cholesterol degradation is hindered by excess of bile acids(1,6), which results in a slight increase of cholesterol concentration, and this in turn retards liver cholesterol synthesis(7).

Due to exchange of cholesterol between serum and tissues, difficulties are encountered in studying *in vivo* rates of cholesterol synthesis in tissues other than liver. Therefore incorporation of acetate-1-C^{14} in serum, liver, kidney and intestine total cholesterol was determined 30 minutes and 6 hours after injection in rats maintained on their respective diets for 21 days. There was no significant change in total cholesterol levels in serum or any tissues, due to dietary cholic acid (Table III). In case of the 6-hour acetate-1-C^{14} incorporation period (Table IV), the cholic acid-treated animals showed about 70% decrease in cholesterol-x-C^{14} concentration in serum and liver; about 50% decrease in kidney, and 20% decrease in the intestine. At end of 30 minute incorporation period, in the cholic acid-treated group, there was about 70% decrease in serum and liver cholesterol-x-C^{14} concentration. However, there was no change in kidney or intestine C^{14} activity.

No difference was observed in levels of liver cholesterol-x-C^{14} 30 minutes or 6 hours after injection of acetate-1-C^{14}. These results agree with the findings of Schwenk *et al.*(8) that metabolism of acetate is essentially completed within 30-60 minutes. In the case of serum cholesterol-x-C^{14}, although percentage decrease effected by cholic acid was the same in both incorporation periods, levels of cholesterol-x-C^{14} at 30 minutes were about one-half those at 6 hours. This is probably due to a finite rate of blood and liver cholesterol exchange.

TABLE III. Total Cholesterol Levels in Serum, Liver, Kidney, and Intestine following 21 Days on 0.5% Dietary Cholic Acid.

Tissue	Total cholesterol: Control	Cholic acid	P values
Serum, mg %	86.0 ±11.0	90.9 ±2.55	.49
Liver, mg/g*	2.68 ± .20	2.72 ± .23	.82
Kidney, " *	4.63 ± .32	4.75 ± .11	.58
Intestine, " *	2.30 ± .24	2.40 ± .24	.63

* Wet wt.

TABLE IV. Cholesterol-x-C^{14} Levels in Serum, Liver, Kidney and Intestine 30 Min. or 6 Hr after Injection of Acetate-1-C^{14} following 21 Days on 0.5% Dietary Cholic Acid.

Tissue	Cholesterol-x-C^{14}, counts/min./mg cholesterol: Control (30 min.)	Cholic acid (30 min.)	Control (6 hr)	Cholic acid (6 hr)
Serum	1,559	421	3,219	893
Liver	3,770	1,092	3,798	1,168
Kidney	71	69	303	141
Intestine	2,076	2,233	3,455	2,815

The exchange of cholesterol between blood and other tissues also explains the *apparent* effect of dietary cholic acid on rate of cholesterol-x-C^{14} synthesis in kidney and intestine at 6 hours, as contrasted with the lack of effect in the 30-minute period. This is more obvious with the kidney because of lower rate of cholesterol synthesis. Absence of any decrease in cholesterol synthesis in kidney and intestine due to dietary cholic acid correlates with lack of effect of dietary cholesterol in these tissues as shown by Gould(9).

Summary. 1. Dietary cholic acid reduced the rate of *in vivo* hepatic cholesterol synthesis within 3 days, as indicated by incorporated acetate-1-C^{14}. 2. Continued feeding of cholic acid produced no further decrease in hepatic cholesterol synthesis rate over the 21-day period. 3. Increased serum bile acid level correlated with decreased liver cholesterol synthesis rates at all time intervals. 4. There was a small but significant increase in liver cholesterol concentration caused by dietary cholic acid. This increase remained constant throughout the experimental period. 5. Dietary cholic acid had no effect on *in vivo* kidney and intestine cholesterol synthesis rates.

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Blood Glutathione and Alloxan Susceptibility in Inbred Mice.* (24503)

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Male mice of the strain Z(C_3H) have been reported(1) to be more resistant to a given intravenous dose of alloxan than are male mice of strain D_8. Degree of alloxan sensitivity of an experimental animal may possibly be related to the endogenous blood and tissue glutathione (GSH) levels, for GSH and other sulfhydryl compounds protect against the diabetogenic effect of alloxan when given prior to alloxan(2,3). Moreover, sodium deficient diet(4) and repeated acetoacetate injection (5) lower the GSH content of the blood and markedly increase susceptibility to alloxan (4,6). Likewise, such factors as cysteine feeding, thiouracil feeding, and thyroidectomy, which increase the liver and kidney GSH level, also increase resistance to alloxan (7). In view of this evidence that factors which alter the GSH content of the blood or tissues alter alloxan susceptibility, it was thought worthwhile to determine blood GSH levels in these 2 strains of mice. These levels were found to differ in the 2 strains, as did alloxan susceptibility, and therefore it seemed important to determine how these characteristics are transmitted to the F_1 hybrids resulting from the cross between these 2 strains.

Material and methods. Adult mice of strains Z(C_3H) and D_8 were used as well as hybrids resulting from crosses between D_8 female and Z(C_3H) male and between D_8 male and Z(C_3H) female. Male and female mice approximately 6 weeks in age were used for GSH determinations, but only male mice were used for alloxan susceptibility studies. Reduced GSH was determined by the alloxan 305 method as described by Patterson and Lazarow(8). Hematocrit levels were determined by the microhematocrit method of Van Allen (9). Glutathione is reported as mg of GSH/100 cc of red blood cells. In the susceptibility studies, alloxan was injected *via* the tail vein using a No. 26 needle, in doses of 40, 60, or 80 mg/kg body weight. Blood sugars were determined by the method of Folin-Malmros (10) at 48 hours and 7 days after the injection. The average blood sugar of normal mice was 135.6 $\pm$ 13.4 mg % 3 hours after the last meal. A level higher than 200 mg % 3 hours after the last meal was regarded as an indication of diabetes.

Results. Table I shows that mice of strain Z(C_3H) (alloxan resistant) had a significantly higher erythrocyte GSH level (39% higher) than those of strain D_8 (alloxan susceptible).

In determining erythrocyte GSH levels of both types of F_1 hybrids, it was found that, regardless of method of crossing; 1) erythrocyte GSH levels were not significantly different from those of Z(C_3H) (alloxan resistant) strain (see Table I), but 2) were significantly

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† Carried out while a medical student, Univ. of Minn. Medical School.

TABLE I. Genetic Control of GSH Level in 2 Strains of Mice (± Stand. Dev.).

Strain	No. of mice	Wt (g)	Hematocrit level (%)	Erythrocyte GSH (mg)	p value (for GSH)
Z (alloxan resistant)	18	17.5 ± .9	44.6 ± 3.2	43.0 ± 7.1	
ZD_8 (F_1 hybrids)	12	17.8 ± 1.4	48.8 ± 2.7	43.4 ± 6.9	.7040
D_8Z (F_1 hybrids)	7	17.8 ± 1.4	51.3 ± 3.9	44.9 ± 6.6	.6312
D_8 (alloxan sensitive)	14	16.9 ± 1.0	48.5 ± 5.1	30.9 ± 6.6	<.001

higher than those of the D_8 (alloxan susceptible) strain. This suggests that the factor(s) responsible for control of erythrocyte GSH level are genetically transmited to the offspring, possibly as a dominant characteristic. However additional studies would be needed to establish this point. When levels of erythrocyte GSH were compared for male and female mice of these strains, no significant difference was found (Table II), suggesting that

TABLE II. Erythrocyte GSH Levels of Males and Females of Two Strains of Mice.

Strain	Sex	No. of mice	Wt (g)	Erythrocyte GSH (mg)	p value (for GSH)
Z	♂	18	15.8	41.5 ± 7.7	.60
Z	♀	13	16.7	40.4 ± 3.9	
D_8	♂	13	15.7	31.5 ± 8.1	.22
D_8	♀	24	14.8	34.4 ± 3.2	

sex does not influence the erythrocyte GSH level.

In alloxan susceptibility studies, initially reported by Martinez *et al.*(1), the Z(C_3H) strain was shown to be more resistant to alloxan than the D_8 strain. In the present study it was observed that F_1 hybrids resulting from crosses between Z(C_3H) and D_8 strains were as resistant to alloxan as was the Z(C_3H) parental strain (Table III). Furthermore, at a given dose of alloxan, blood sugar levels of the diabetic group of both the Z(C_3H) strain and of hybrids were similar (Table III). These studies suggest that the factor(s) which controls alloxan resistance appears to be genetically transmitted to the F_1 hybrids.

Discussion. These findings imply a relationship between erythrocyte GSH level and resistance to intravenous alloxan in these inbred strains of mice, supporting the hypothesis that the endogenous GSH level is related to the alloxan susceptibility.

Since the erythrocyte GSH level differed in these inbred strains of mice, it alone might determine their differing susceptibility to alloxan. In this case, since GSH is not found in the blood plasma(11,12), alloxan would have to enter the red blood cells and be detoxified there. It seems probable however, that GSH levels within the B-cell of the islets of Langerhans may also determine the resistance to diabetogenic agents. Therefore, it is important to measure the sulfhydryl level in the B-cells since it is these that are selectively destroyed by administered alloxan(13), possibly because of their deficiencies in GSH(14).

In considering all factors determining alloxan resistance, the level of erythrocyte GSH should be taken into account as possibly determining the extent of detoxification of the diabetogenic agent before it reaches the B-cell. The susceptibility to diabetogenic agents, therefore, is probably due to a number of factors, one of which is erythrocyte GSH.

Summary and conclusions. Studies on

TABLE III. Alloxan Susceptibility in 2 Strains of Mice and Their F_1 Hybrid Crosses.

	% of mice developing diabetes when inj. with specified dose of alloxan (mg/kg)			Blood sugar of diabetic mice when inj. with specified dose of alloxan (mg/kg)			Erythrocyte GSH level of mouse strain used (mg)	
Strain of mouse*	40	60	80	40	60	80	GSH	Stand. dev.
D_8	25	100	100	291	382	417	30.9	6.6
Z (C_3H)	0	58	91		292	358	43.0	7.1
Hybrid crosses	0	75	93		305	357	43.9	7.1

* 10-14 mice were used in each group for alloxan inj.

erythrocyte GSH were carried out using 2 inbred strains of mice and their F_1 hybrids. 1. The erythrocyte GSH level in the $Z(C_3H)$ (alloxan resistant) strain and the F_1 hybrid was significantly higher than it was in the D_8 (alloxan susceptible) strain. 2. The $Z(C_3H)$ strain and the F_1 hybrids were equally resistant to alloxan, whereas the D_8 strain was significantly more susceptible to alloxan. 3. Thus, both the blood GSH level and the degree of alloxan susceptibility also appears to be genetically controlled.

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Effect of Volatile Fatty Acids, Sodium and Potassium Bicarbonate in Purified Diets for Ruminants.*† (24504)

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A previous report(1) showed that growth of lambs fed a purified diet containing a mixture of glucose and salts of acetic, propionic and butyric acid was superior to that of lambs fed diets containing a mixture of either glucose and starch or glucose, starch and cellulose. After a period of 2 years, all 3 animals on glucose-starch diet and 2 of 3 animals on cellulose diet died, whereas 5 of 6 animals on volatile acids diet survived. Further evidence for the adequacy of "acids" diet was furnished by the fact that the 5 survivors were normal in weight and in appearance. The present study was undertaken to investigate the factor(s) responsible for the superiority of volatile-fatty-acids diet. Data obtained from 2 experiments are presented.

Procedures.‡ Details of biological procedures were the same as those described previously(1) except that in this study all lambs were restricted to amounts they would consume in 3 feedings daily. Prior to placing the lambs on experiment, they were treated with *Clostridium perfringens* type D bacterin vaccine to prevent enterotoxemia. Volatile fatty acids were determined by Keeney's method (2) on samples of rumen fluid taken by stomach tube 6 hours after feeding, during latter part of first experiment. In Exp. I, diets 2, 4, 5 and 6 shown in Table I were tested. Diet 2, used as positive control, has the same formulation as the "complete" volatile acids diet used previously(1); diet 4 is similar to 2 except that Na butyrate is omitted, and diets 5 and 6 correspond to 2 and 4, respectively, except that triacetin is substituted for acetate salts. Ten lambs averaging 31 lb initially were assigned to the 4 diets; 2 lambs to each of diets 2 and 4, and 3

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TABLE I. Purified Diets Used in Investigation.

Components	Diet 1	Diet 1 (Na + K)	Diet 2	Diet 4	Diet 5	Diet 5 (Na + K)	Diet 6
	%						
Casein	30	26.5	30.0	30.0	30.0	26.5	30.0
Glucose	36	31.8	24.1	27.7	27.7	24.5	24.1
Corn starch	20	17.7					
Hydrog. veg. oil	4	3.5	4.0	4.0	4.0	3.5	4.0
Na acetate			9.0	9.0			
K "			8.5	8.5			
Mg "			3.0	3.0			
Na propionate			3.0	3.0	3.0	2.7	3.0
Ca "			4.8	4.8	4.8	4.2	4.8
Na butyrate			3.6				3.6
Triacetin					20.5	18.1	20.5
$KHCO_3$		4.4				4.4	
$NaHCO_3$		7.3				7.3	
Vitamin mixture*	5	4.4	5.0	5.0	5.0	4.4	5.0
Mineral "	5†	4.4†	5.0‡	5.0‡	5.0§	4.4§	5.0§

* Vitamin mixture (5 lb): thiamine HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine HCl, 570 mg; folic acid, 57 mg; p-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione (2-methylnaphthoquinone), 115 mg; 0.1% B_{12} in mannitol, 4.66 g; alpha tocopherol acetate, 570 mg; glucose, 2132 g. Administered 4000 I.U. of A and 400 I.U. of D/day/100 lb body wt via capsules.

† Mineral mixture (5 lb): $CaHPO_4$, 818 g; KCl, 273 g; NaCl, 239 g; $MgSO_4$, 204 g; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg; $CoCO_3$, 9 mg; KI, 6 mg; glucose, 722 g.

‡ Mineral mixture (5 lb): Cu, Fe, Mn, Zn, Co and I salts same as in (†). NH_4Cl, 138 g; H_3PO_4 (85%), 226 g; glucose, 1892 g.

§ Mineral mixture (5 lb): Cu, Fe, Mn, Zn, Co and I salts same as in (†). KCl, 273 g; $MgSO_4$, 204 g; NH_4Cl, 138 g; H_3PO_4 (85%), 226 g; glucose, 1415 g.

Grateful acknowledgment is made to Buckeye Cotton Oil Co., Cincinnati, O., for hydrogenated vegetable oil (Primex B and C); to E. I. duPont de Nemours and Co., Wilmington, Del., for Ca and Na propionate; to Union Carbide Chemical Co., Charlotte, N. C., for Na and K acetate; to R. P. Scherer Corp., Detroit, Mich., for vit. A and D; to Hoffmann-LaRoche, Nutley, N. J., for biotin; and to Merck and Co., Rahway, N. J., for other vitamins.

TABLE II. Averages of 24-Week Weight Gains and Feed Intakes of Lambs in Exp. I.

Diet No.	Triacetin	Na butyrate	Feed intake, lb	Wt gain, lb
2	0	+	141	39 (2)*
4	0	0	227	51 (2)
5	+	+	108	+2 (3)
6	+	0	105	−1 (3)

* No. in parentheses = No. of lambs receiving diet.

to each of diets 5 and 6. Exp. II was conducted in 2 parts. The first consisted of 2 replications of diets 4, 1(Na + K) and 5 (Na + K), the latter 2 diets being identical to diets 1, described previously(1), and 5, respectively, except for inclusion of Na and K bicarbonates. The second part involved 2 more replications of the diets in part I and 2 replications of diets 1 and 5 (Table I). Sixteen lambs averaging 27 lb were used.

Results. Lambs fed triacetin-containing diets, 5 and 6, gained little or none during 24 weeks, indicating that triacetin was not a satisfactory substitute for acetate salts (Table II). The subsequent death of 2 lambs in each group gave further evidence of the inadequacy of these diets. Lambs on diet 4 consumed more and gained more than lambs on diet 2, but feed efficiencies were similar, *e.g.*, 4.45 and 4.65 lb of feed/lb of gain, respectively. Percentage distribution of ruminal volatile acids was similar for all diets (Table III). Comparison of C_4 acid fractions shows that butyrate was present in rumen

TABLE III. Mean Fatty Acid Composition of Rumen Fluid from Lambs in Exp. I.

Diet No.	Samples analyzed	Acid fractions, molar % of total C_2	C_3	C_4	C_5	C_6 and higher
2	9	52.5	21.1	12.4	5.0	8.9
4	8	49.1	23.4	12.8	6.3	8.4
5	8	46.7	23.4	14.0	7.1	8.8
6	5	45.2	23.4	16.6	7.3	7.6

TABLE IV. Averages of Weight Gains and Feed Intakes of Lambs in Experiment II.*

	Part 1		Part 2		Avg, parts 1 + 2
Diet No.	Feed intake, lb	Gain, lb	Feed intake, lb	Gain, lb	Gain, lb/wk
1			41	−1.0 (1)‡	
1 (Na + K)	159	39.5 (2)†	69	13.8 (2)	1.9 (4)
4	151	36.3 (2)	60	11.5 (2)	1.7 (4)
5			32	−1.3 (2)	
5 (Na + K)	123	27.0 (2)	71	17.5 (2)	1.7 (4)

* Part 1 covers period of 16 wk and Part 2 a period of 10 wk.
† No. in parentheses = No. of lambs receiving diet.
‡ One of the two animals on diet 1 died after 5 wk on experiment.

samples in about the same ratio irrespective of presence of butyrate in the diet.

These findings agree with observations from a supplementary study with mature sheep and calves fed purified diets either without dietary voltaile acids or with a supplement of either propionate alone or acetate alone. Irrespective of diet the acid fractions shown in Table III were always present, and ratios of C_5 and lower fractions were remarkably similar among different diets.

The results of Exp. II, Table IV, show that the over-all gains of lambs fed diets 1 (Na + K) and 5 (Na + K) are equal to those fed control diet 4. Feed efficiencies were also similar, *e.g.,* 4.08, 4.43 and 4.37 lb feed/lb gain for diets 1(Na + K), 4, and 5 (Na + K), respectively. The poor performance of animals fed diets 1 and 5 in part 2 confirms earlier results(1) and furnishes additional evidence showing the beneficial effect on growth of adding Na and K bicarbonates to the diets.

Discussion. The results of the first experiment showing similar patterns of ruminal fatty acids irrespective of diets fed substantiate reports(3,5,6) indicating that volatile fatty acids are formed in the rumen from a variety of substrates. These data probably account for the finding that dietary volatile fatty acids were not the limiting factor of the purified diets investigated. Our results suggest that bulk or roughage is not an essential in the diet of ruminants and that a purified diet devised for simple-stomach animals is suitable for ruminants provided it is adequately supplemented with Na and K cations in the form of bicarbonate. The role of these cations in the purified diets is most probably associated with the buffering capacity in the rumen.

With a normal roughage diet, as volatile acids are formed, they are neutralized by buffers of the saliva, which is high in Na bicarbonate(4). Mineral cations of saliva entering the rumen are reabsorbed maintaining the alkaline reserve of blood and becoming again available for recirculation by saliva. Thus, a fixed amount of buffer can be used to neutralize a much larger amount of acid. McDougall(4) estimated that the CO_2 content of saliva is about 4 times the normal alkali reserves of sheep's blood and cited Zuntz's observation that amount of alkali secreted each day in the saliva of oxen was about 6 times that contained in blood. Purified diets fed here consist of readily fermentable substances, and therefore, when fed to the ruminant, the ruminal acids would be formed more quickly than on a normal diet(3), thus requiring more buffering capacity at a given time. Furthermore, since animals fed purified diets did not ruminate appreciably, flow of saliva was probably reduced markedly(4), thereby decreasing ruminal buffering capacity of the animal. Under these circumstances, the appetite of the lambs is depressed by an adaptive mechanism to maintain a physiologically compatible environment in the rumen. Conceivably the role these buffers play in the ruminal environment also effects shifts in population of rumen microorganisms and in quantities and rate of transport of end products which in turn affect productive energy of the diet.

Summary. Two experiments with lambs were conducted to determine the dietary essentials supplied by various purified diets. In

the first experiment triacetin could not replace acetate salts in the diet and butyrate could be omitted. Butyrate was found present in the same amount in the rumen contents irrespective of its presence in the diet. The second experiment showed growth could be obtained on simplified diets supplying energy as carbohydrate or triacetin if sodium and/or potassium were added to the ration.

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Role of Sodium in Production of Myocardial Necroses by Stress.* (24505)

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Previous experiments have shown that, following prolonged pretreatment with large doses of certain corticoids, *e.g.*, 2α-methyl-9α-chlorocortisol (Me-Cl-COL), a brief period of neuromuscular effort (induced by forced restraint) can elicit acute, massive myocardial necroses in the rat(1). Subsequent investigations revealed that the precipitating effect of neuromuscular effort is not specific, since a variety of other stressors (hot or cold baths, surgical trauma, adrenaline) also produce myocardial necroses after conditioning with Me-Cl-COL. It was found, furthermore, that certain sodium salts (phosphates, sulfates, perchlorate) can, while others (chloride, acetate, citrate, lactate) cannot replace the stressors in precipitation of myocardial necroses after Me-Cl-COL conditioning(2). No salts of cations other than Na were effective, but these observations show that the anion also plays an important role in the pathogenesis of this type of myocardial necrosis. We were particularly impressed by the fact that, although NaCl and the organic Na-salts were almost always totally ineffective after Me-Cl-COL-pretreatment, they occasionally produced very pronounced cardiac necroses, in rats damaged through fortuitous circumstances. It was, therefore, postulated that, after suitable sensitization with corticoids, certain Na-salts (phosphates, sulfates, perchlorate) produce cardiac necroses under ordinary conditions, while others (chloride and several organic salts) are inactive in themselves, but become highly effective during stress. We now wish to report upon experiments which substantiate this concept.

Materials and methods. Two hundred female Sprague-Dawley rats, with mean initial body weight of 100 g (range: 95-110 g), were subdivided into 20 equal groups, as indicated in Table I. Me-Cl-COL† was administered subcutaneously, in the form of its acetate, as a microcrystal suspension of 100 μg in 0.2 ml of water, once daily. All the Na-salts were given by stomach tube, at the dose of 2 mM, in 2 ml of water, twice daily. Neuromuscular stress was induced by maintaining the rats on a board with adhesive tape, for a period of 17 hours, on the fifth day of the experiment. The animals of all groups were killed with chloroform on the sixth day. The hearts were fixed in neutral formalin and embedded in paraffin for subsequent staining with our fuchsin technic(2). The intensity of the necroses was assessed in terms of an arbitrary scale of 0-3, and the means of these readings (with standard errors) are listed in Table I.

Results. It is evident from our data that, in these rats, cardiac necroses do not occur

* Supported by grants from Gustavus and Louise Pfeiffer Research Fn. and from Geigy Pharmaceuticals.

† Generously supplied by Upjohn Co.

TABLE I. Role of Sodium in Production of Myocardial Necroses by Stress in 20 Groups.

Treatment	Cardiac necroses
None	0
NaCl	0
Na-Acetate	0
-Citrate	0
-Lactate	0
Me-Cl-COL	0
+ NaCl	0
+ Na-Acetate	.1 ± .10
+ -Citrate	0
+ -Lactate	.2 ± .20
Restraint	.1 ± .10
+ NaCl	.1 ± .10
+ Na-Acetate	.2 ± .20
+ -Citrate	.1 ± .10
+ -Lactate	.1 ± .10
+ Me-Cl-COL	.4 ± .18
+ " + NaCl	1.9 ± .14
+ " + Na-Acetate	2.6 ± .30
+ " + -Citrate	2.1 ± .25
+ " + -Lactate	3.0 ± .0

spontaneously (Group 1), or after treatment with any of the Na-salts (Groups 2-5) or Me-Cl-COL alone (Group 6). Treatment with Me-Cl-COL + Na-salts (Groups 7-10), restraint alone (Group 11) or restraint + Na-salts (Groups 12-15) produced only minute necrotic foci in occasional animals. These lesions were never large enough to be detectable by the naked eye or the hand loupe. Combined treatment with restraint + Me-Cl-COL (Group 16) was somewhat more effective, but (owing to the low dosage of Me-Cl-COL) even here the lesions were very small and only occasionally visible under loupe magnification. By contrast, in the animals treated with restraint + Me-Cl-COL in combination with the otherwise ineffective Na-salts (Groups 17-20), pronounced cardiac necroses were invariably detectable by naked-eye inspection, because they affected large areas of the myocardium. Deaths occurred only among the rats treated with restraint + Me-Cl-COL combined with either Na-citrate (40% mortality, Group 19) or Na-lactate (10% mortality, Group 20).

Discussion. The principal outcome of these experiments is that certain Na-salts (ineffective in themselves and during simultaneous treatment with either Me-Cl-COL or a stressor) become highly effective in producing massive myocardial necroses, when given in combination with Me-Cl-COL and a stressor, such as forced restraint. Further studies will be necessary to elucidate the mechanism through which otherwise ineffective Na-salts can produce acute myocardial necroses in Me-Cl-COL-conditioned rats during restraint. It is also still uncertain whether there exists any close pathogenetic relationship between the experimental cardiac necroses thus produced and the acute cardiac infarcts that occur in man. It is significant, however, that both these conditions can be elicited by an acute stress situation.

Summary. Certain Na-salts (chloride, acetate, citrate and lactate) are well tolerated even by rats simultaneously treated with 2α-methyl-9α-chlorocortisol (Me-Cl-COL) or a severe stressor, such as forced restraint. However, these same salts produce massive and sometimes fatal myocardial necroses, in rats exposed to the stress of forced restraint after conditioning with Me-Cl-COL. It appears that during stress the metabolism of certain otherwise innocuous Na-salts and/or steroids is so altered that they acquire severe cardiotoxic properties.

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Pituitary ACTH and Plasma Free Corticosteroids Following Bilateral Adrenalectomy in the Rat.*† (24506)

CLAUDE FORTIER

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From a study of changes in pituitary ACTH, plasma free corticosteroids and adrenal weight induced in the rat by a surgical procedure (splenectomy), it was concluded (unpublished) that the initial postoperative depletion of pituitary ACTH, and its subsequent rise, reflected opposite effects of stress and corticosteroids on release of the hormone. The present report on the effect of hypocorticoidism on ACTH release and synthesis was based on concurrent determinations of pituitary ACTH and plasma free corticosteroids, following bilateral adrenalectomy in the rat.

Materials and methods. Male Sprague-Dawley rats (175-225 g, initial B.W.) were acclimatized for 2 weeks to laboratory conditions (constant lighting and temperature (25 ± 1°C); Purina Fox-Chow and water *ad lib.*) and distributed into equal numbers of experimental and control groups of 3 subjects of uniform weight. *Bilateral adrenalectomy* was performed by lumbar approach, under ether anesthesia. The animals were maintained postoperatively on 0.9% NaCl drinking solution, and killed by decapitation at progressively later intervals from onset of anesthesia. They were closely examined postmortem for presence of ectopic or regenerated adrenal tissue, and discarded in the few instances when such tissue was detected. The *adenohypophyses* were collected, pooled and processed, as previously described,¶ for ACTH assay by *in vitro* technic of Saffran and Schally(1). The results, computed and tested for validity according to Bliss' methods for factorial analysis and analysis of variance (2), were expressed in milliunits (mu) of ACTH/mg of fresh tissue (with confidence limits (antilog $C^2M \pm CtSm$) for odds of 19 in 20), and in percentage of mean control level. Equal emphasis was placed on the internal (*within* assays) and external (*between* assays) variances of the combined estimates (unweighted logarithmic means) by computing their standard errors from the averages of crude and unweighted $S\bar{m}$'s. In a separate experiment performed under strictly identical conditions, *arterio-venous blood* from neck vessels was collected into heparinized beakers for plasma free corticosteroid determination by modification(3) of Silber's fluorometric method(4). The results, expressed in corticosterone equivalents, as well as in percentage of the control baseline, were corrected for fluorescence of unknown origin, observed to persist in blood of adrenalectomized(4,5) and hypophysectomized(5) rats, by subtracting 5 (a value corresponding to this fluorescent residue) from the original estimates.

Results. Pituitary ACTH response to bilateral adrenalectomy (Fig. 1) was poly-

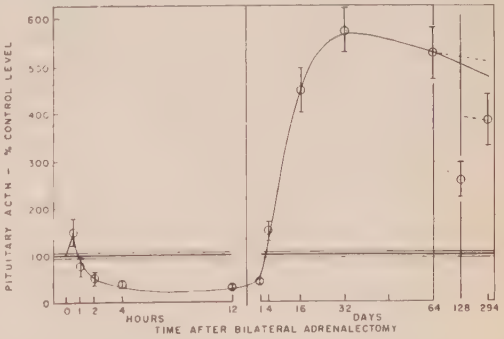

FIG. 1. Effect of bilateral adrenalectomy on pituitary ACTH concentration. Dots correspond to means of 3 assays for the first time ordinate, of 2 for the others; control baseline, to mean of 18 assays. In this as in following figure, stand. errors are respectively indicated by T-shaped bars for experimental, and parallel lines for control, values.

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† The author is indebted to Miss R. C. Wood for able technical assistance, and to Drs. Eleanor J. MacDonald, Univ. of Texas Anderson Hospital and Tumor Inst., and Karl Hopkins, Univ. of Oregon Medical School, for advice pertaining to statistical treatment of the data.

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TABLE I. Effect of Bilateral Adrenalectomy on Pituitary ACTH Concentration.

Interval	ACTH/mg fresh tissue (mu)	95% confidence limits (mu)	Index of precision (λ)†
0			
Mean of 18 assays $\pm S.E.$*	40.6 ± 1.7		.105 ±.009
30 min.	89.8	28.3–282.2	.168
	40.0	25.2– 63.6	.091
	62.4	33.3–218.2	.143
	60.7 ± 12.9		
1 hr	39.8	13.6–122.4	.169
	23.6	7.9– 47.9	.148
	30.7 ± 7.8		
2	15.7	4.3– 26.9	.125
	28.3	11.9– 53.0	.132
	21.1 ± 5.7		
4	13.8	6.0– 21.6	.090
	19.4	12.3– 27.9	.060
	16.4 ± 2.6		
12	11.6	4.1– 19.1	.102
	13.7	2.3– 27.2	.171
	12.6 ± 1.9		
1 day	15.8	10.6– 21.2	.057
	19.4	3.0– 38.8	.174
	17.5 ± 2.4		
4	54.9	30.6–148.7	.131
	69.2	45.3–126.5	.094
	61.7 ± 8.6		
16	173.5	119.5–272.4	.082
	192.0	64.5–865.8	.182
	182.4 ± 19.9		
32	226.4	153.2–399.3	.082
	241.2	151.3–512.7	.104
	233.3 ± 20.8		
64	197.6	118.4–305.6	.094
	234.0	160.7–392.5	.081
	214.8 ± 21.9		
128	97.4	52.3–152.3	.099
	112.5	68.4–227.5	.110
	104.7 ± 13.6		
294	142.8	78.1–305.7	.125
	172.6	101.6–668.2	.132
	157.0 ± 22.5		

* Stand. errors ($S.E.$'s) of combined estimates (unweighted logarithmic means) were computed from averages of their crude and unweighted $S\bar{m}$'s.
† Stand. dev. (s) of response (Y) divided by slope (b) of assay: $\lambda = s/b$ (2).

phasic: transient rise within first 30 minutes, followed by fall to 40% of control level after 4 hours, slower rate of decline and stabilization at low level for the next 20 hours, subsequent rise, to a crest of 575% on 32nd day, and slow regression thereafter to lower, though still markedly elevated, values. The mean angle of decline from the 64th day was computed from values recorded after 128 and 294 days. An identical pattern characterized the 2 series of determinations carried out independently (Table I).

Free corticosteroids (Fig. 2) disappeared from the plasma within 4 hours. The residual fluorescence, inherent to the method(3,4) and corresponding to 5 γ of corticosterone/100 ml of plasma (uncorrected scale), was remarkably constant thereafter, as shown by standard error of 0.1, computed from all determinations in chronic groups.

Discussion. A slight increase of pituitary ACTH concentration, following bilateral adrenalectomy in the rat, was recently reported by Kitay *et al.*(6) on the basis of assays at a single postoperative interval (7 days), by modification of Saffran and Schally's technic. Previous and more detailed studies were based on Sayers' assay(7). From single point determinations, in which the potency of the unknown in terms of adrenal ascorbic acid-depleting activity was computed directly from a standard log dose-response chart, Gemzell *et al.*(8) reported that bilateral adrenalectomy induced a progressive fall of pituitary ACTH to a minimum of 30% of the preoperative level after 24 hours. This was followed by a rise to 178 and 191% of the control values, after 7 and 21 days respectively. For lack of determinations before the first hour, the initial rise in

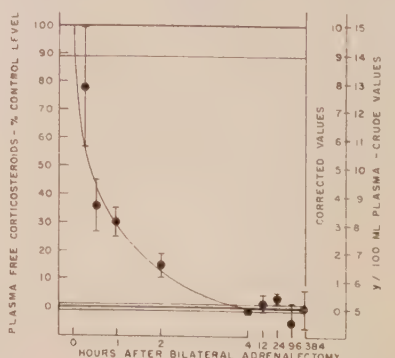

FIG. 2. Disappearance of plasma free corticosteroids, following bilateral adrenalectomy. Dots and control baseline respectively correspond to means of 4 and 42 determinations.

concentration, in the present study at the 30-minute postoperative interval, was not detected. Percentagewise, the results of Gemzell *et al.* are in fair agreement with ours, with respect to the subsequent 24-hour depletion, but differ markedly thereafter. In view of the utilization by this group of a uniform aliquot (0.01 mg of dry pituitary) throughout their series, the comparatively low values recorded at 7- and 21-day p.o. intervals may be attributed to improper adjustment of the unknown to the useful range (rectilinear portion of log dose-response slope) of Sayers' assay.‡ The same may hold, to a lesser extent, with regard to the low post-adrenalectomy values (114, 214 and 254% after 7, 14 and 18 days respectively) reported by Sydnor and Sayers (9) on the basis of 5-point assays, in which samples from adrenalectomized animals were uniformly determined at half the concentration used for controls.

The amplitude of the observed changes in pituitary ACTH concentration provides clear evidence of a dissociation between rates of ACTH release and synthesis. A constant level of pituitary ACTH admittedly corresponding to a steady state, defined by equal rates of synthesis and release, increasing and decreasing levels will be respectively indicative of a predominance of synthesis over release and *vice versa*. Given similar trends of storage and release, the trend of synthesis would be readily available through algebraic summation. The interpretation of our data is facilitated, in this regard, by recent observations of Brodish and Long(10). Using a cross-circulation technic, in which the adrenal ascorbic acid (AA) response of the hypophysectomized recipient to 20 ml of circulated blood from the donor, indicated the latter's relative blood ACTH level(11), these investigators reported that bilateral adrenalectomy resulted, within 15 minutes, in a significant rise of blood ACTH which gradually subsided until the 12th hour, when minimal values were recorded. A secondary rise to supernormal values was observed thereafter. It levelled off, after 2 weeks, until the end of observation period (4 weeks). Notwithstanding the limitations of the cross circulation technic,§ it is noteworthy that similar time relationships characterize succession of events initiated by adrenalectomy at blood and pituitary levels. In both instances, the initial phase of the reaction involves immediate rise followed by a fall to minimum level after 12 hours, the secondary phase, a subsequent rise to supernormal levels. It would thus appear that, following a transient burst of synthesis, reflected by concomitant initial peaks of pituitary and blood ACTH, a predominant, though gradually receding, acceleration of rate of release accounts for pituitary ACTH depletion, a greater and sustained acceleration of synthesis, for its secondary rise.

A comparison of pituitary ACTH responses to bilateral adrenalectomy and to splenectomy, 2 forms of surgical trauma respectively characterized by rapid disappearance and equally rapid rise of blood corticosteroids, illustrates the latter's involvement in adrenocorticotrophic regulation. No initial rise in concentration was observed after splenectomy. A fall of lesser amplitude (40, as compared to 70% after adrenalectomy) was followed by a rise to a transient peak, sharply contrasting in magnitude and duration with the upward phase of the response to adrenalectomy. From available indices of ACTH release, it appears furthermore that, whereas the fall can be ascribed, in both instances, to a predominantly accelerated rate of release, the secondary rise is related to depressed release after splenectomy, and to predominant synthesis after adrenalectomy. It may be inferred therefrom that 1) the ACTH-releasing effect of stress, common to the 2 experimental conditions, is markedly influenced by the level of circulating adrenal cortical hormones, and that 2) withdrawal of these hormones enhances both release and synthesis of

‡ This range reportedly extends from 0.15 to 2.5 mu of ACTH/100 g, B.W.(8), as compared to 3-300 mu/100 mg adrenal, for Saffran and Schally's assay(2).

§ Adrenals of recipient animal being already stimulated (17% AA depletion) by the ACTH released as a result of cannulation of donor(10,11), this technic provides an unusually narrow margin of sensitivity to additional stimulation.

ACTH, but has a predominant effect on the latter. The eventual arrest and reversal of this reaction may be tentatively related to the intervention of a complementary feed-back mechanism of a more sluggish type, as suggested by Gemzell and Heijkenskjöld's observation of a delayed and cumulative depressing effect of exogenous ACTH on the synthesis of the hormone in the adrenalectomized animal(12).

Summary. Pituitary ACTH and plasma free corticosteroids were concurrently determined following bilateral adrenalectomy in the rat. Free corticosteroids disappeared from the plasma within 4 hours. Pituitary ACTH rose to 150% of the control level within the first 30 minutes, fell to 40% after 4 hours, to 30% after 12, stabilized at this level for the next 12 hours, rose from then on to supernormal levels, up to a crest of 575% on the 32nd day, and regressed, thereafter, to lower, though still markedly elevated, values. From a correlation of these findings with available data on blood ACTH levels under comparable conditions, it is inferred that, following a transient burst of synthesis reflected by the initial peak, a predominant, though gradually receding, acceleration of the rate of release accounts for the pituitary ACTH depletion, a greater and sustained acceleration of synthesis, for its subsequent rise. It is further suggested that the ACTH-releasing effect of stress is markedly influenced by the level of circulating adrenal cortical hormones, and that withdrawal of these hormones enhances both release and synthesis of ACTH, but has a predominant effect on the latter.

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Effect of Hydrocortisone on Pituitary ACTH and Adrenal Weight in the Rat.*† (24507)

CLAUDE FORTIER

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From previous study of changes in pituitary ACTH concentration induced, in the rat, by bilateral adrenalectomy(1), it was inferred that withdrawal of the adrenal cortical hormones enhances both release and synthesis of ACTH, but has a predominant effect on synthesis. As a correlate, the present report on effect of hypercorticoidism on adrenocorticotrophic activity was based on concurrent determinations of pituitary ACTH and adrenal weight during hydrocortisone administration in the rat.

Materials and methods. Male Sprague-Dawley rats (175-225 g, initial B.W.) were acclimatized for 2 weeks to constant lighting

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† The author is indebted to Miss R. C. Wood for valuable technical assistance.

and temperature (25 ± 1°C); Purina Fox-Chow and water *ad lib.*; and distributed into equal numbers of experimental and control groups of 3 subjects of uniform weight. *Hydrocortisone* (Hydrocortone Acetate, Merck; Saline suspension, 25 mg/ml) was administered at 6 mg/100 g, I.P. on first day, in Exp. A and B; at 3 and 6 mg/100 g, S. C. on subsequent days in Exp. A and B respectively. Antibiotics (Combiotic, Pfizer; Crystalline Penicillin G. Procaine, 200,000 U and Crystalline Dihydrostreptomycine, 250 mg/ml of saline suspension) were administered (0.2 ml/100 g, S.C.) on alternate days throughout treatment. Animals were killed by decapitation at progressively later intervals from first injection of hydrocortisone. *Adenohypophyses* were collected, pooled and processed, as previously described,¶ for ACTH assay by *in vitro* technic of Saffran and Schally(2). The results, computed and tested for validity according to Bliss' methods for factorial analysis and analysis of variance(3) were expressed in milliunits (mu) of ACTH/mg of fresh tissue (with confidence limits (antilog $C^2M \pm CtSm$) for odds of 19 in 20), and in percentage of mean control level. Equal emphasis was placed on internal (*within* assays) and external (*between* assays) variances of combined estimates (unweighted logarithmic means) by computing their standard errors from the averages of crude and unweighted $S\bar{m}'s$(3). *Adrenal glands* were dissected free of fat and weighed to the nearest 0.05 mg. To dissociate growth from treatment effects, results were expressed as percentages of mean control values for each time interval.

Results. An immediate, slight (14%) and short-lived (2 hr) depletion‡ of *pituitary ACTH* (Table I, Fig. 1A) was followed by rise to a peak of 133% of initial level, reached after 4 hours and tapering off slightly in the next 20 hours. A steep, though somewhat irregular, decrease in concentration, bearing no

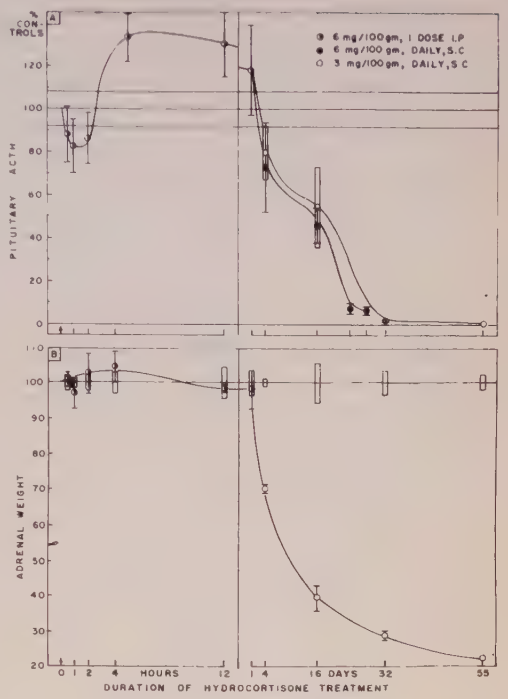

FIG. 1. Effect of hydrocortisone on pituitary ACTH concentration (A) and adrenal wt (B). Black & white and monochrome dots, in A, respectively correspond to means of 2 assays, with *S.E.*'s computed from avg of crude and unweighted $S\bar{m}$'s, and to single assays, with *S.E.*'s derived from internal *Sm*'s. Control baseline was obtained from mean of 13 assays. Dots and their respective control baselines, in B, correspond, for all but 55-day time ordinate (1 experimental value), to means of 4-6 determinations.

significant relationship to dosage, was observed thereafter. Less than 10% of the original concentration was found after 24 days of the 6 mg regimen. No ACTH could be detected, within limits of sensitivity of the assay (less than 1.5 mu/mg of pituitary, when sample was assayed at 4 times the usual concentration) after 55 days of hydrocortisone administration at the 3 mg dosage level. This regimen (3 mg/100 g, daily) induced an exponential reduction of the *adrenal weight* to hypophysectomy level (10-14 mg, corresponding to 22-34% of controls)§ within 32-55 days. Hydrocortisone administration also resulted, from onset, in complete arrest of

¶ Fortier, C., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 628.

‡ Though none of the values corresponding to first 3 time ordinates differs significantly from the control mean, their grouping is suggestive of a true fall.

§ Nearly identical values were recorded by Harris and Jacobsohn(4) 42-45 days after hypophysectomy in male rats.

TABLE I. Effect of Hydrocortisone on Pituitary ACTH Concentration.

Interval	ACTH/mg fresh tissue (mu)	95% confidence limits (mu)	Index of precision (λ)†
	Exp. A§		
0			
Mean of 7 assays ±*S.E.**	84.0 ± 7.9		.120 ±.015
30 min.	65.3	38.9–144.4	.115
1 hr	55.7	43.5– 73.1	.054
2	62.3	45.9– 89.4	.066
4	105.7	63.8–282.8	.113
12	95.2	63.2–167.9	.084
1 day	76.0	55.6–114.1	.068
4	66.9	45.7–107.7	.083
16	46.2	12.4–139.0	.175
32	1.6	1.0– 2.2	.065
55‡	0 (<1.5)		
	Exp. B‖		
0			
Mean of 6 assays ±*S.E.**	65.2 ± 4.7		.100 ±.020
30 min.	65.4	40.0–127.6	.108
1 hr	67.4	53.7– 87.9	.038
2	65.8	38.6–146.0	.103
4	91.9	76.7–113.0	.034
12	93.3	67.4–145.5	.065
1 day	100.3	85.3–121.1	.031
4	48.2	22.3–117.3	.144
16	30.2	17.4– 44.1	.082
24‡	4.7	1.1– 8.4	.107
28‡	4.2	1.3– 7.8	.077

* Stand. errors (*S.E.*'s) of combined estimates (unweighted logarithmic means) were computed from avg of their crude and unweighted $s\bar{m}$'s.

† Stand. dev. (s) of response (Y) divided by slope (b) of assay: $\lambda = s/b$ (3).

‡ Sample obtained from 1 donor, instead of usual 3.

§ Hydrocortisone acetate, 6 mg/100 g, I.P. on first day; 3 mg/100 g, S.C. on subsequent days.

‖ Hydrocortisone acetate, 6 mg/100 g, I.P. on first day, S.C. on subsequent days.

growth which is admittedly related to the protein catabolic effect of glucocorticoids(5,6). The few animals which survived beyond the first 30 days of treatment evidenced marked cachexia.

Discussion. The foregoing observations on pituitary ACTH-depleting effect of chronic hydrocortisone administration are in general agreement with previously reported results of cortisone administration. From direct comparisons of the adrenal ascorbic acid-discharging activity of pituitaries from intact and cortisone-treated (100 mg, daily) dogs, Farrell and Laqueur(7) reported that the ACTH content was depressed to 54, 3 and 2% of the normal, after 14, 44 and 71 days of treatment. Using similar procedure in rats, Michailova (8) observed a reduction to 61 and 55%, after 19 and 24 days of cortisone (2.5 mg, daily) administration. More recently, Kitay *et al.*(9), who used a modification of Saffran and Schally's assay, found a 41% depletion, in the rat, after 7 days of treatment with cortisone (5 mg, daily). No previous observation is available on the immediate effects of steroid injection, displayed, in the present study, by a transient fall and a subsequent rise of the pituitary ACTH concentration.

Interpretation of the observed changes in pituitary ACTH concentration is largely dependent on information provided by indices of ACTH release. Thus, from adrenal ascorbic acid (AA) depletion noted one hour after cortisone injection in the rat(10), taken in conjunction with the reported blockade of the AA response to stress, from hydrocortisone administration 4 hours prior to its onset(11), it may be inferred that the initial post-injection fall of pituitary ACTH is related to ACTH-releasing effect of the injection *per se*, and its subsequent rise, to inhibitory effect of the steroid on further release. Sustained and complete blockade of release during the chronic phase of treatment is evidenced by the exponential decline of adrenal weight to hypophysectomy level. Were release and synthesis the only factors to be considered, total inhibition of synthesis could be inferred from failure of pituitary ACTH to rise under these conditions. Its very fall, however, points to involvement of a third factor: inactivation of the stored hormone. Whether the observed rate of ACTH disappearance (half-life of 5.2 ± 3.5 days, computed from regression of x (time) on y (log concentration)) corresponds to inactivation alone or in opposition to residual synthesis, cannot be ascertained from our data.

Summary. Pituitary ACTH and adrenal weight were concurrently determined, in the rat, during treatment with hydrocortisone (3 and 6 mg/100 g, daily). An immediate and slight depletion of pituitary ACTH was fol-

lowed by rise to a peak of 133% of the initial level, reached after 4 hours and tapering off in the next 20 hours. A sharp decrease was observed thereafter. Less than 10% of the original concentration was found after 24 days of the 6 mg regimen. No ACTH could be detected after 55 days, at the 3 mg dosage level. This regimen (3 mg) induced an exponential reduction of the adrenal weight to hypophysectomy level within 32-55 days. From available indices of ACTH release under comparable conditions, it is inferred that the initial post-injection fall of pituitary ACTH is related to the ACTH-releasing effect of the injection *per se*, and its subsequent rise, to the inhibitory effect of the steroid on further release. Sustained and complete blockade of release during the chronic phase of the treatment is suggested by the rate of adrenal weight loss. The concomitant disappearance of pituitary ACTH is imputed to inactivation of the stored hormone in the absolute or relative absence of synthesis.

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Lipid Composition of Human Serum Lipoprotein Fraction with Density Greater than 1.210.* (24508)

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On ultracentrifugation of samples of untreated human serum, Turner *et al.*(1) found that the fraction of high density at the bottom of the tube contained phospholipid and neutral fat in concentrations approximating those of whole serum, but little cholesterol. Subsequently, Turner *et al.*(2) estimated that approximately 65% of the serum phospholipid was present in a lipoprotein containing no free cholesterol. Hillyard, Entenman, Feinberg and Chaikoff(3) reported that on ultracentrifugation of human serum, the density of which had been raised to 1.220 by addition of potassium bromide, a fraction sedimented which contained an average of 13% of the total serum phospholipid and small quantities of cholesterol. Using more vigorous centrifugation of serum with density raised to 1.21, Havel, Eder and Bragdon(4) sedimented a fraction which contained lipid phosphorus but practically no cholesterol. They estimated that such a lipoprotein, which varied little in concentration between healthy subjects and patients with hyperlipemia, could account for no more than 15% of the total serum phospholipid. The lipid (ethanol-acetone soluble) phosphorus, which was not dialyzable against 0.15 M sodium chloride, was precipitable with serum proteins by zinc hydroxide, soluble in water, insoluble in petroleum ether, and migrated in starch electrophoresis largely with the α_1 globulin-albumin fraction. Similarities of this phosphorus fraction and a serum phos-

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TABLE I. Phospholipid Composition of Lipoprotein of Density Greater than 1.210.

Patient	Sex	Age	% total lipid P				% of total serum lysolecithin in 1.210 fraction	% of total lipid P in 1.210 fraction	Total serum lipid P, μMole/ml
			"Cephalin"	Lecithin	Sphingomyelin	Lysolecithin			
E.R.	♂	22	3	23	7	68			2.90
N.R.	♂	23	5	31	15	49	52	9.0	2.58
H.D.	♂	24	7	21	17	55	66	7.5	2.70
A.G.	♂	39	3	22	6	70	61	8.3	3.06
R.D.	♀	31	9	30	18	43	48	8.0	4.01
A.R.	♀	31	11	20	19	50	50	6.0	3.30
Mean			6	25	14	56	55	7.8	3.09
S-1*	♂		5	20	13	63			
S-2*	♂		2	22	5	70			
S-3*	♂		3	27	9	60		9.3	3.31
S-4†	♂		6	33	14	48		5.7	3.07

* Nonfasting sample from healthy young adult male.
† Pooled nonfasting sample from 2 healthy young adult males. Density, 1.262.

phopeptide (containing no phospholipid) described by Hack(5) were pointed out, and Bragdon, Havel, and Boyle(6) postulated that the serum fraction sedimented at density 1.21 contained no true lipid other than unesterified fatty acids. In the present study, the ultracentrifugal method of Havel, Eder, and Bragdon(4) was used to isolate the serum fraction with density greater than 1.210, and the lipid extract of this fraction was chromatographed on silicic acid(7,8). Evidence was found for the presence of phospholipid and triglyceride, but little, if any cholesterol. The phospholipid, which comprised about 8% of the total serum phospholipid of 5 healthy young adults, was composed predominantly of lysolecithin.

Materials and methods. Serum samples were obtained from 4 male and 2 female white adults in the fasting state and from 5 young male white adults 4 to 5 hours after a light breakfast (Table I). All 11 subjects were apparently healthy. Ultracentrifugation of serum was begun within 2 to 3 hours after withdrawal according to the method of Havel, Eder, and Bragdon(4). Samples were centrifuged at 105,000 x g at 15°C in the #40 rotor of a Model L Spinco ultracentrifuge. In the series of fasting subjects, 5 to 8 ml of serum were diluted to a density of 1.019 using a NaCl-KBr solution of density 1.085(4), centrifuged for 22 hours, and the top layer removed. A known amount of the remaining sample was then diluted to density 1.210 using NaCl-KBr solution of density 1.346(4) and centrifuged for 48 hours. The bottom layer, which contained lipoprotein of density greater than 1.210, was then extracted for lipid analysis. In one of the non-fasting subjects (S-1, Table I), 10 ml of serum was made to a density of 1.019, centrifuged for 22 hours, and the upper layer removed; the bottom layer was made to a density of 1.063, centrifuged for 22 hours, and the upper layer removed; the bottom layer was then made to a density of 1.210, centrifuged for 48 hours, and the bottom layer extracted. In the other subjects, 9 ml of serum was made directly to a density of 1.210 (S-2, S-3, Table I) or 1.262 (S-4, Table I) with the solution of density 1.346, centrifuged for 48 hours, and the bottom layer extracted. In all these samples, centrifugation resulted in a distinct layer at top and bottom of tube with a colorless zone, about one-half the length of the tube, in between. The sedimented serum fraction with density greater than 1.210 was always visibly clear. The fractions were separated by slicing the tube at approximately the center of the colorless zone with a Spinco tube slicer. Centrifuged serum samples were extracted with methanol-chloroform (1:1) and the extracts chromatographed on silicic acid columns (1 g), to determine concentrations of individual phospholipids, cholesterol, cholesterol esters, and triglycerides(8). In fasting

subjects, these same determinations were done on the centrifuged fractions with density less than 1.210 and on whole serum (to be published). In 2 (S-3, S-4, Table I) of the nonfasting subjects, only total serum lipid phosphorus was determined, to calculate the percentage of total lipid phosphorus present in the fraction with density greater than 1.210. The lipid extract of centrifuged serum was also chromatographed on silicic acid-impregnated filter paper (Whatman #3) using 20% methanol in chloroform (v/v) as solvent. In addition, column fractions were hydrolyzed in 6N HCl at 110° for 18 hours in a sealed tube and chromatographed on filter paper (Whatman #1) using butanol-acetic acid-water (4:1:4, using butanol layer) as solvent. These paper chromatograms were developed by (1) spraying with ninhydrin in butanol, followed by heating at about 110° for 5 minutes and (2) the phosphomolybdic acid stain of Chargaff, Levine and Green(9). The column fractions were analyzed for phosphorus by the method of Fiske and Subbarow(10), for cholesterol and cholesterol esters by the method of Schoenheimer and Sperry(11) as modified by Sperry and Webb(12) and for ester bonds by the method of Rapport and Alonzo(13). The quantities of phosphorus in the "cephalin" and sphingomyelin fractions were usually so small that the errors in the phosphrous determinations were of the order of ± 30%.

Results. In fasting normal subjects, lipid phosphorus of serum fraction with density greater than 1.210 comprised about 8% of total serum lipid phosphorus (Table I). About one-half of this lipid phosphorus appeared to be in the form of lysolecithin; and the lysolecithin in this fraction in turn accounted for about one-half of total serum lysolecithin. The other phospholipid constituents, identified according to mobility on column, were lecithin, sphingomyelin, and "cephalin," in order of decreasing concentration. Some variation in concentrations of these phospholipids between the subjects may be attributed to errors in determination of the small amounts of phospholipid chromatographed and, possibly, to the level at which the centrifuge tube was sliced. Centrifuging the serum at a density of 1.262 instead of 1.210 seemed to have little effect on phospholipid composition of this fraction (Table I).

The lysolecithin fraction in 2 samples tested had an ester-to-phosphorus ratio of 0.9 and had the same mobility as the lysolecithin of whole serum lipid extract when chromatographed on silicic acid-impregnated filter paper with methanol-chloroform elution. Acid hydrolysis of this material followed by filter paper chromatography with butanol-acetic acid-water elution gave rise to one spot with the phosphomolybdic acid stain with the mobility of choline and very small amounts of ninhydrin-reacting material. When 0.01 micromoles (on basis of phosphorus) of this fraction were incubated with washed fresh sheep red blood cells at 37.5°C for 2 hours, visible hemolysis was evident.

Concentrations of cholesterol and cholesterol esters, which were eluted initially off the column(8), were too small to be measured. Less than 0.2 mole of total cholesterol/mole of phosphorus appeared to be present. Total ester, however, presumed to be triglyceride, could be measured and fell within a fairly narrow range, averaging about one mole of triester per 2 to 3 moles of phosphorus.

Discussion. The evidence presented indicates that the fraction of centrifuged human serum with density greater than 1.210 contains phospholipid and non-phospholipid ester, presumably triglyceride, with little or no cholesterol, either free or esterified. The high density of this fraction, the low water solubility of most lipids present, lack of opalescence in this fraction, and the demonstration that the lipid migrates with α_1-globulin-albumin in starch electrophoresis(4,14) make it clear that the lipid in this fraction is bound to protein. The high density, moreover, suggests a low lipid-to-protein ratio. Whether all of these lipids are attached to one protein in the proportions found cannot be stated, although lack of discernible difference in lipid composition of a fraction of density greater than 1.262 as compared to one of density greater than 1.210 was suggestive of this possibility.

This lipoprotein fraction was unusual not

only in its content of little or no cholesterol, but because about one-half of the phospholipid appeared to be in the form of lysolecithin. The lysolecithin in this fraction in turn accounted for about one-half of the total serum lysolecithin. The properties of the ethanol-acetone soluble phosphorus found in this fraction by Havel, Eder, and Bragdon (4) are consistent with those of lysolecithin (15). The high density ethanol-ether soluble phosphorus described by Hack (5) as a phosphopeptide with its phosphorus in the form of serine phosphate, however, may be a different substance.

The percentage of total serum phospholipid in this fraction is in general agreement with the values of Havel, Eder, and Bragdon (4). It is conceivable, however, that the density of this lipoprotein is a function of its triglyceride content, since in this fraction the triester-to-phosphorus ratio varied little between samples. If this is the case, it is possible that more of this lipoprotein could be isolated by ultracentrifugation at a serum density somewhat less than 1.210 and greater than 1.125, as Havel, Eder, and Bragdon (4) showed that at 1.125 density other lipoproteins apparently sediment. If all serum lysolecithin were present in this type of lipoprotein, moreover, serum concentration of this lipoprotein would be double, as about one-half of serum lysolecithin was found in serum fractions with density less than 1.210, including fractions with densities less than 1.019, 1.019 to 1.063, and 1.063 to 1.210 (to be published).

Although no metabolic role can be ascribed as yet to this lipoprotein fraction, Turner *et al.* (2) reported that phosphorus turnover in the lipoprotein system without cholesterol appeared to be greater than in the system with cholesterol.

Summary. A human serum lipoprotein fraction with density greater than 1.210 has been separated by ultracentrifugation and found to contain about 8% of total serum phospholipid, non-phospholipid ester, presumably triglyceride, and little or no cholesterol. About one-half of the phospholipid was found to be in the form of lysolecithin, which in turn comprised about one-half of the total lysolecithin of serum.

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Adrenal Responses of California Quail Subjected to Various Physiologic Stimuli.* (24509)

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The well established pituitary-adrenal relationship in mammals is still poorly defined in birds(1). There are indications that the avian pituitary-adrenal axis may not be, in every sense, the physiologic equivalent of its mammalian counterpart. Several workers have noted the relative refractoriness of the avian adrenal(2,3,4) and have suggested that a certain degree of autonomy may be characteristic of the bird adrenal (interrenal) gland. The purpose of our experiments was to determine, using both wild and pen-raised California valley quail, the adrenal responses elicited by factors known to result in alteration of the adrenal cortex in laboratory mammals.

Materials and methods. A total of 67 California valley quail, *Lophortyx californica*, was used. Of these, 32 were wild birds and 35 were pen-raised. Birds were placed, 2/cage, in wire cages measuring 22″ x 14″ x 12″. Generally, a male and female were placed together. With the exception of uncaged control birds, each group of quail was caged 7 days. Then birds were aged, weighed to nearest gram (subsequently corrected for weight of food in the crop), decapitated, and thyroids, adrenals and spleen removed and fixed in 10% neutral formalin. Coloration of primary coverts was used as criterion for aging (5). Adrenals were cleaned of connective tissues, and weighed out of formalin on a 0-50 mg Roller-Smith balance.

Wild and pen-raised birds were divided into 5 main groups, each totalling about a dozen quail. These groups were (1) controls (taken directly from field or game farm and killed immediately), (2) caged controls (caged and sham-injected), (3) cold-treated, (4) cortisol-treated, and (5) ACTH-treated. Wild and pen-raised components of one experiment were not run simultaneously, but as near the same time as possible. Caged control birds were sham-injected with 0.2 ml of 0.9% saline/day for 7 days. All injections were intramuscular. Cold-treated birds were maintained at 40°F for 7 days. Cortisol-treated birds were injected with 1 mg of cortisol acetate in 0.2 ml saline daily for 7 days. ACTH-treated birds were injected with 1 unit of Upjohn Corticotropin in 0.2 ml 16% gelatin-1% thymol solution daily for 7 days. Dosages chosen would be expected to induce adrenal changes in laboratory mice, if given proportionately to body weight (Bern and Nandi, personal communication). Left adrenals, with exception of some birds which died, were embedded in paraffin, sectioned at 8 μ and stained with hematoxylin and eosin. For each sectioned adrenal, 50 measurements of width of interrenal cords were made with ocular micrometer (at 400 x) and average cord width computed.

Results. Weights of individual adrenals revealed no consistent difference between left and right glands. No consistent sex difference in combined adrenal weight or adrenal weight/body weight ratio (AW/BW) was found, nor was any definite evidence for seasonal changes found. Histologic examination of the quail adrenal revealed that groups of chromaffin cells are scattered throughout the interrenal tissue. These islands of chromaffin cells vary in size and shape not only in different areas of same gland, but also from gland to gland. Interrenal tissue is arranged in cords generally 2 cells thick, that loop and branch throughout the entire gland.

The pen-raised caged control group had a mean combined adrenal weight and mean AW/BW ratio significantly greater than wild caged control group. The mean AW/BW ratio of the pen-raised ACTH-treated group was significantly higher (at 1% level) than that

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TABLE I. Adrenal Weight and Histometric Data for Pen-raised and Wild California Quail.

Treatment	No. of quail	Adrenal wt (l + r) in mg	Adrenal wt, mg / Final body wt, g	Interrenal cord width, μ
Pen-raised				
Uncaged control	6*	11.6 ± 1.2	.069 ±.009	26.7 ± .8
Caged control	11	17.7 ± 1.1‡	.116 ±.003‡	29.4 ± .4‡
Cold	6	15.2 ± 1.0	.095 ±.007§	27.2 ±1.0
Cortisol†	3	13.2 ± 1.2§	.091 ±.015	27.0
ACTH	6	19.0 ± 1.2	.140 ±.011§	28.6 ± .8
Wild				
Uncaged control	6	15.8 ± 1.5	.087 ±.006	28.4 ± .8
Caged control	8	14.3 ± .8	.090 ±.008	29.0 ±1.1
Cold	5	13.7 ± .2	.086 ±.003	28.6 ± .6
Cortisol†	4	11.4 ± 1.4	.088 ±.008	25.5
ACTH	5	16.7 ± .9	.102 ±.001	29.9 ± .4

* Weight data from 1 bird excluded because adrenals were partially fused.
† Included some animals that died during experiment, but received at least 5 cortisol inj.
‡ Significantly different from respective uncaged controls at 1% level of confidence.
§ *Idem* caged 5%

of wild ACTH-treated birds (Table I). Adrenal data from all caged control birds were compared with those from all cortisol-treated birds. No significant difference in mean AW/BW ratios was found, but cortisol-treated birds had a significantly smaller mean combined adrenal weight and mean cord width. Comparing combined adrenal data from all caged controls with those from all ACTH-treated quail, no significant differences were seen between mean values. To test the significance of the difference between the 2 possible extremes in the experimental series, data from all ACTH-treated birds were compared with those from all cortisol-treated birds. The mean AW/BW ratio, mean combined adrenal weight, and mean cord width were greater in ACTH-treated birds, the differences being significant at the 5%, 1%, and 5% levels respectively.

Spleen weights and spleen weight/body weight ratios (SW/BW) were so highly variable that no attempt was made to treat the data statistically. No consistent or significant difference was found between the SW/BW ratios of pen-raised and wild groups. In addition, the mean epithelial height of thyroid follicles from wild and pen-raised cold-treated quail was not found to vary significantly from those of the respective caged control birds.

Discussion. There is evidence that the avian adrenal cortex possesses some activity independent of pituitary control(2,3). Recent work(4) on domestic chickens indicates that hormones secreted by the bird adrenal elicit responses similar to those in the mammal. However, it was believed that the bird adrenal may be relatively autonomous and that it may function at a relatively high level even in the absence of the anterior pituitary.

Various treatments to which our quail were exposed are known to elicit definite changes in adrenals of laboratory mammals. The main limitation of this study involves the comparatively small number of birds used. Nevertheless, the lack of consistent differences between experimental and control groups, particularly in the case of cortisol-treated birds, and the failure of cold treatment to activate the adrenal, indicate that the mechanisms involved here may differ somewhat from those known to operate in mammals. It is concluded that these treatments did not provide sufficiently intense stimuli to cause comparable pituitary-adrenal activation. Several investigators have demonstrated direct relationship between cold environmental temperatures and thyroid activation(6,7,8,9). However, in the quail, cold treatment (7 days at 40°F) was again not a sufficiently intense stimulus to cause pituitary-thyroid activation.

Summary. Adrenal responses of pen-raised and wild California valley quail subjected to caging, cold, cortisol, and ACTH

treatments have been studied and compared. Groups of pen-raised and wild birds showed no consistent nor significant differences in either adrenal weight or histometry. Spleen weights were highly variable. No histologic evidence of thyroid activation was seen in birds subjected to cold. Experimental treatments, potentially capable of causing marked adrenal responses in common laboratory mammals, were relatively ineffectual in eliciting adrenal response in the quail. It is suggested that the adrenal, and possibly the thyroid, may be comparatively non-reactive in quail as compared with laboratory mammals.

The author is indebted to Drs. A. S. Leopold, H. A. Bern, and L. W. Taylor for encouragement and suggestions.

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Mating Types of *Paramecium bursaria* from Japan.* (24510)

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Paramecium bursaria is known to include 6 varieties(1,2,3). These varieties may differ greatly in geographical distribution; some are widely distributed, others are relatively restricted. Variety III, for example, is highly restricted; during many years of collecting in this country, this variety has been found in only 3 localities, 2 in North Carolina, one in Massachusetts(1). Varieties VI and I, on the other hand, are widely distributed. Variety VI has been found in England, Ireland and Czechoslovakia; and Variety I in many parts of United States, China(4), and as reported here, apparently in Japan also. Members of any one of these 6 varieties do not, as a rule, mate with members of any of the other varieties. Within each variety, animals of diverse mating types conjugate readily; animals of the same mating type do not conjugate. Variety I, found in the United States and China, and apparently Japan, contains 4 mating types designated A, B, C, and D. Variety II, found in United States, contains 8 mating types, designated E, F, G, H, J, K, L, and M. Variety III, also found in United States, contains 4 mating types designated N, O, P, and Q. Varieties IV and V were found in Russia. Variety IV is known to have 2 mating types, designated R and S. Only one mating type (mating type T) has so far been found in Variety V. Further collections from Russia would probably yield other mating types in this variety. Variety VI, in Czechoslovakia, England and Ireland, is known to contain 4 mating types designated U, V, W, and X. Thus, there are now known in this species of Paramecium 6 varieties containing 23 mating types. Reported here are results of investigation on *P. bursaria* collected in Japan. The material included the following collections: Two, one from Kyoto, the other from Tokyo, sent by Dr. Shozo Inoki of Osaka Univ., Osaka, in Feb. 1953; these 2 collections yielded 16 clones. A collection sent by Dr. Toshiko Watanabe of Ashiya

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TABLE I. Collections of *Paramecium bursaria* in Japan.

Date received	Collector	Locality	No. of clones isolated	Mating types found
Feb., 1953	Shozo Inoki	Kyoto	8	B
"	"	Tokyo	8	D
Jan., 1954	Toshiko Watanabe	Ashiya City	11	D
Apr., 1958	S. Hayashi	Sapporo	18	B, C
"	K. Tanaka	Nagoya	17	A, C, one clone immature?
May, 1958	C. Tanaka	Miyagawamura, Takigun, Mieken	3	A, C, one clone adolescent

City, in Jan. 1954, yielding 11 clones. A collection sent by Dr. S. Hayashi of Hokkaido Univ., Sapporo, Apr. 1958, yielding 18 clones. A collection by Dr. K. Tanaka of Nogoya in the same month, yielding 17 clones. A collection by Dr. C. Tanaka of Miyagawamura, May 1958, yielding 3 clones. These 65 clones obtained from Japan are listed in Table I.†

Methods. The animals were cultured essentially as described by Jennings(1,5) and by Sonneborn(6), in a lettuce infusion inoculated with *Aerobacter cloacae*. When the clones were kept for extended periods they were stored in culture tubes 200 mm × 25 mm with screw caps, placed in racks near windows with northern exposure, as described by Chen (4). The Japanese clones conjugated readily with American and Chinese clones of Variety I, but not with varieties II and VI. They were therefore considered to belong to Variety I. The mating types of the Japanese clones were determined by mixing them with known 4 mating types A, B, C, and D of Variety I, of Chinese origin. Thus, if a Japanese clone did not mate with known mating type A animals, but did mate with B, C, and D animals, it was considered a mating type A clone. Similarly, a Japanese clone not mating with B animals but mating with A, C, and D animals was considered a B clone. In mixtures of a Japanese clone and a Chinese clone of appropriate mating type of Variety I, agglutinative mating reaction and pair formation occurred in normal manner. Many pairs were formed in such a mixture. Occasionally 3 or 4 animals were observed conjugating in the manner reported by Chen(7).

Results. All 8 clones from Kyoto belonged to mating type B. All 8 clones from Tokyo belonged to mating type D. All 11 clones from Ashiya City belonged to mating type D. Some of the 18 clones from Sapporo belonged to mating type B, others to mating type C. Of the 17 clones from Nagoya, 16 belonged to mating types A and C; one clone was apparently immature since it did not mate with any other clone, and upon its arrival from Japan was isolated from a vial found to contain many conjugating pairs. Of the 3 clones from Miyagawamura, one belonged to mating type A, one to mating type C, and one was adolescent, forming only a few pairs with mating types B and C, and none with A and D(5).

Summary. Studies on 65 clones of *Paramecium bursaria* collected in Japan showed that these animals apparently belonged to Variety I. All four mating types in this variety: A, B, C, and D were found.

† It is a pleasure to acknowledge my indebtedness to these biologists.

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Suppressive Effect of Anticholinergic, Oxyphenonium Bromide, on Reserpine-Stimulated Gastric Secretion.* (24511)

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Relatively small doses of reserpine have been shown to act as a stimulant to gastric hydrochloric acid production(1,2). This effect has been observed both after oral and parenteral administration of the drug(3,4). Although the site of action of reserpine is unknown, its effect on gastric secretion may be attributable to an alteration of sympathetic-parasympathetic balance, resulting in parasympathetic predominance(5). If the parasympathetic effect is a function of dosage, as has been suggested(5), it may possibly be altered by administration of anticholinergic drugs. Studies in dogs have shown that increased secretion of hydrochloric acid induced by reserpine can be eliminated by subcutaneous administration of the anticholinergic drug, oxyphenonium bromide(6). For this reason, oxyphenonium bromide was chosen as the anticholinergic agent for this study.

Methods. Eleven healthy young adults, 22 to 38 years old, were selected. The investigation was divided into 3 study periods during which the drugs were administered orally in 4 equally divided doses daily as follows: The first study period consisted of 16 experiments on 11 subjects who recived oxyphenonium bromide daily for 7 days following a control period. In 11 experiments they were given 20 mg and in 5, 40 mg daily. Since there was no statistically significant difference between the 2 groups ($P>.5$) they were considered as one and listed in the second column in Fig. 1 as "oxyphenonium bromide 20 mg." The second group consisted of 8 subjects who, after a control period, received 0.5 mg of reserpine daily for 7 days. Following 2 weeks of rest they received for 7 days a combination of 0.5 mg of reserpine and 20 mg of oxyphenonium bromide daily. In the third group 7 subjects received .32 mg of reserpine daily for one week, rested for 2 weeks, and were then given a combination of .32 mg of reserpine and 20 mg of oxyphenonium bromide daily for another 7 days. These groups served as their own controls, and each period of drug administration was followed by a 2-week recovery period. Gastric contents of fasting subjects were aspirated during 4 consecutive 15-minute intervals prior to and on the final day of each period of drug administration. Gastric analyses were performed 2 hours after administration of reserpine and oxyphenonium bromide. This 2-hour interval was chosen to take advantage of the peak activity of these 2 agents. The action of reserpine on gastric acid production is apparent 60 to 90 minutes after oral administration and persists for 4 or more hours(3). With oxyphenonium bromide, a depression of gastric secretion occurs within 30 minutes of oral administration, and maximum effect persists from 1 to 5 hours(6,7). The free hydrochloric acid concentration and volume were measured and hourly production of free hydrochloric acid was calculated for each period.

Results (Fig. 1). The first 2 columns of the figure reveal that when 20 mg of oxyphenonium was administered, hourly production of free hydrochloric acid diminished significantly below control values ($P<.05$).

The next 3 columns in the figure reveal that when 0.5 mg of reserpine was given, free hydrochloric acid production increased significantly ($P<.08$), but when 20 mg of oxyphenonium bromide was added, hydrochloric acid production dropped significantly ($P<.02$). This drop in hydrochloric acid was even below control levels ($P<.05$).

Similar results were recorded in the last 3 columns of the figure. Free hydrochloric acid production rose significantly above control values after .32 mg of reserpine ($P<.05$) and

* The supply of reserpine and oxyphenonium bromide (Antrenyl®) and a grant-in-aid from Ciba Pharmaceutical Co., Summit, N. J. are gratefully acknowledged.

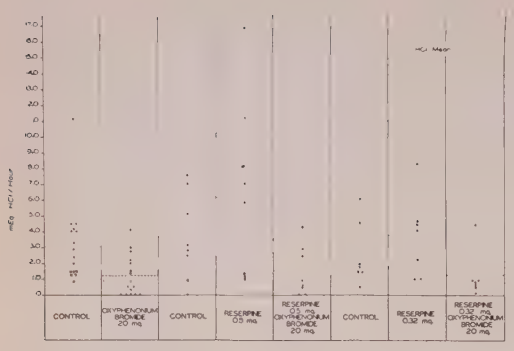

FIG. 1. Comparison of effects of orally administered reserpine and oxyphenonium bromide on human gastric secretion. • represent individual experiments.

dropped with a combination of .32 mg of reserpine and 20 mg of oxyphenonium ($P < .01$). Values obtained with this combination were found to be significantly lower than those of the controls ($P < .05$).

Discussion. Reserpine is believed to act centrally by altering the autonomic homeostatic mechanism(1). Its net effect can be explained on the basis of partial suppression of sympathetic activity at the hypothalamic level. The resultant parasympathetic predominance may explain the increased gastric secretion noted in reserpine-treated subjects. The observation that oxyphenonium bromide reduced gastric hydrochloric acid production in reserpine-treated subjects is in agreement with Plummer's study in dogs(1). The observation is further supported by a recent report from Krogsgaard, who was able to suppress reserpine-induced gastric hypersecretion by concomitant administration of oxyphenonium bromide in 2 of 5 patients who were given the drugs parenterally(8). Oxyphenonium bromide acts by blocking postganglionic cholinergic nerve transmission, and by blocking the impulses to the parietal cell may antagonize the effect of reserpine at the effector cell level(6).

No side effects were encountered with these agents, except for varying degrees of nasal stuffiness after larger dose of reserpine.

Summary. An anticholinergic drug, oxyphenonium bromide, (20 mg/day) was administered concomitantly with reserpine (.32 mg and .5 mg/day) to normal young adults. Addition of anticholinergic compound suppressed the rise in gastric free hydrochloric acid induced by administration of reserpine alone and reduced the gastric acidity below control levels in all instances.

We are indebted to Dr. Calvin Zippin for statistical analysis of the data.

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Life Span of Rat Erythrocytes as Determined by Cr^{51} and Differential Agglutination Methods. (24512)

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Estimates of life span of rat erythrocytes have ranged from 4 to 100 days. The 2 most common methods used to obtain these estimates are measurement of return of circulating erythrocytes to normal levels after hemorrhage, induced erythropenia by chemical agents, or hypoxic erythremia, and disappearance of isotopically labeled erythrocytes from circulation. The literature cited by Hall *et al.* (1) indicates that in most cases the first method gave lower estimates (4 to 18 days) and the second, higher values (45 to 100 days). They, however, using Cr^{51} as erythrocyte label, reported average survival to be ~24 days with a half life of 8 days. A similar half life was reported by Aschkenasy *et al.*(2) who also used the Cr^{51} method. By using the Cr^{51} method, the survival time of Sprague-Dawley rat erythrocytes was ~60 days(3). Fairly reliable estimates of erythrocyte life span are obtainable by the Ashby technic(4), in which transfused cells are detected by differential agglutination or hemolysis. Although comparison of Cr^{51} and Ashby technics indicated that the former method is satisfactory for estimating erythrocyte life span in man and dog(5-8), a similar comparison has not been made in the rat. Owen's experiments(9) indicated that a differential agglutination technic could be used to determine life span of rat erythrocytes. He followed the rate of disappearance of exchanged cells after rats of distinguishable serotype had been disjointed from parabiosis. We used the same markers in the CD strain furnished by Owen and found that it is possible to transfuse a rat possessing D type erythrocytes with C cells and, at various intervals thereafter, test the blood for percentage of C type cells by means of anti-C serum (agglutinin) produced in rabbits. In this paper we compared rat erythrocyte survival curves obtained by the differential agglutination and Cr^{51} methods.

Methods. Blood was withdrawn from the abdominal aorta of C donors into heparin-containing syringes and was incubated with $Na_2Cr^{51}O_4$† (5.0 μc/ml of whole blood) for ~ 1 hr at room temperature with frequent swirling. About a third of blood volume was withdrawn from the jugular vein of D rats, which were then immediately transfused with a restorative amount of whole C blood‡ containing Cr^{51}-labeled erythrocytes. Two tail blood samples were obtained at 24 hrs and thereafter at 6 intervals up to the 62nd day. One sample (~20 μl) was tested with anti-C serum for percentage of C type cells as described (10). The other sample (20 or 50 μl) was taken for radioactivity determination. Erythrocytes obtained for radioactivity determination at 24 hrs were washed with 100 volumes of saline, centrifuged, and resuspended in 2 ml of distilled water. Subsequent blood samples were not washed but were discharged directly into test tubes containing 2 ml of distilled water. A well-type scintillation counter was used to determine radioactivity of the samples. Counting efficiency was about 35% and counting error about 2%. Considerable variation was present in percentages derived from the agglutination method because this technic is subject to errors inherent in any procedure involving erythrocyte counting. In addition, the smaller the number of agglutinable cells present, the larger the possible error. On the other hand, a C cell concentration as small as 2% can be detected among D cells as was determined on dilution series of known mixtures of the 2 kinds of cells.

Results. In individual survival curves of C

*Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

† Abbott Labs., specific activity 20 μc/μg.

‡ Total blood vol. was calculated as 5½% of body weight; donor blood was not pooled.

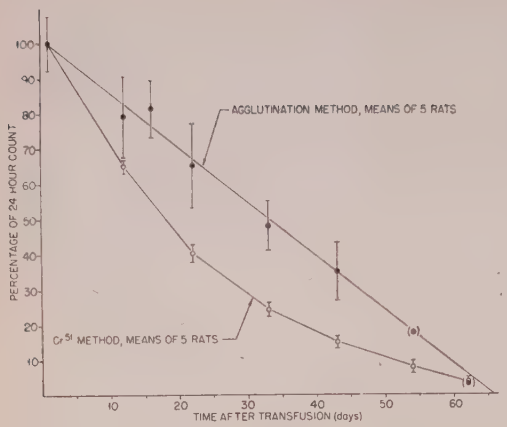

FIG. 1. Survival of rat erythrocytes simultaneously determined by agglutination and Cr^{51}-labeling technics. Vertical bars indicate $\pm$ one stand. error of the mean [S.E. $= \sqrt{\Sigma d^2/n(n-1)}$]. Agglutination points in parentheses are estimates based on observations in counting chamber rather than on numerical counts. Counting of C and D cells in artificial mixtures showed that 2% C cells was just detectable. When agglutination was stronger but still not countable (<10%) the value of 5% was arbitrarily assigned.

erythrocytes in 5 D recipients, determined by agglutination technic, the C cells disappeared linearly from circulation of recipients until they were just detectable 61 days after injection. At this time they comprised about 2 to 5% of the circulating population in each rat. No C cells were detected on the 68th day. One day later the same 5 D rats were injected with C erythrocytes labeled with Cr^{51}. Fig. 1 gives survival curves obtained both from agglutination and Cr^{51} methods. C type erythrocytes again disappeared in a linear fashion from the recipients' circulation and were just detectable on the 62nd day. On about the 33rd day 50% of the initial population of C type erythrocytes remained. An estimate of the extinction time was 65.7 days based on a fitted line calculated from the agglutination data obtained up to day 43. Although the 95% confidence interval for this estimate was wide (42.5 to 114.1 days), the estimated percentage of C type cells on days 54 and 62 indicated that the extinction time was close to 65 days.

In contrast, the survival curve obtained for Cr^{51}-labeled C erythrocytes was curvilinear rather than linear. By day 62 ~3% of the initial radioactivity was present (Fig. 1). On day 17 half of the initial radioactivity was present. Radioactivity values ploted on semilogarithmic paper gave a straight line for the first 45 days.

Discussion. Donor erythrocytes disappear curvilinearly from a recipient's circulation when factors producing random destruction are present and disappear linearly in absence of such factors(11). Brown and Eadie(12) reported that random destruction occurred in healthy cats and rabbits and occasionally in dogs when blood was tagged with Fe^{55}. Our results suggest that C type cells in D recipients disappeared in a linear manner, indicating that random destruction of rat erythrocytes does not normally occur. The disappearance curve of the Cr^{51}-labeled erythrocytes was curvilinear and showed time relations similar to those obtained for Sprague-Dawley rats(3). Although the 50% survival time obtained by the isotope and agglutination methods differed, the extinction points derived from these methods both occurred at about 65 days. Similarly, extinction time as derived from both methods is about the same for erythrocytes of man(5-7), and the dog(8). In the absence of random destruction, the curvilinear line obtained by plotting the Cr^{51} data reflects elution of the Cr^{51} from the erythrocytes. Calculation of the elution rate according to a formula used by Stohlman and Schneiderman(8) indicates that the isotope was lost from rat erythrocytes at the rate of 1 to 2% per day for the first 45 days, a rate that compares favorably with those obtained by others in dog and man. Precise estimate of erythrocyte life span is, however, precluded by variations in the elution rate. Cr^{51} apparently does not have a toxic effect on erythrocytes, since, according to agglutination data, similar survival patterns were obtained whether or not the transfused cells were labeled.

Recipient D rats we used were transfused on 3 different occasions with C type erythrocytes whose survival was determined by means of differential agglutination. Results of second and third experiments have been discussed (Fig. 1). In the first experiment, how-

ever, transfused cells in 3 rats had reached low levels by 15th day. Immunization against C erythrocytes was not indicated since a secondary response was not elicited by a second or third transfusion of C type cells. The CD strain of rats also segregate for independently inherited red cell antigens (E and F) both of which are isoantigenic (R. D. Owen, personal communication). It is possible that shortened survival in the 3 rats of the first experiment resulted from production of antibodies against these or other unidentified red cell antigens. Failure to observe "immune clearance" in the second and third transfusion may then reflect the absence of corresponding isoantigens in donors.

Our data suggest that the Cr^{51} method gives a reliable estimate of life span of rat erythrocytes when the extinction point is used, although the method gives a falsely shortened 50% survival time because of chromium elution. The life span we obtained compares well with that reported by Berlin *et al.*(13) and Owen (personal communication) but is considerably longer than that reported by Hall *et al.*(1). Differences in technic could partly account for the variance since these workers obtained the first blood sample 1 hr after transfusion, whereas we used a 24-hr period. The relative merits of each method are discussed by Eadie and Brown(11).

Summary. Survival of rat erythrocytes has been studied by simultaneous use of 2 methods, Cr^{51}-labeling and differential agglutination. Results suggest that random destruction of rat erythrocytes did not occur and that the extinction of radioactivity of Cr^{51}-labeled cells is a valid criterion for determination of erythrocyte life span. Both methods suggested a life span of about 65 days.

The authors wish to thank Dr. A. W. Kimball for statistical analysis of the results.

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Immunologic Treatment of Tumors.*† (24513)

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We have prepared anticancer antibodies by injection of tumor cells into animals of a different species, and have injected the antibody solution into host bearing a tumor. Antibodies prepared in rabbits against Walker 256 rat carcinosarcoma were injected into rats at the same time they were inoculated with 10,000 Walker 256 cells. The recent work of Hiramoto and Nungester(1), Korngold and van Leenwen(2) among others showed the feasi-

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bility of producing antibodies against certain tumors; Korngold and associates showed the reactive sites *in vivo* by means of electrophoretic and isotopic studies. It was believed that isolation of large amounts of concentrated gamma and alpha-2-globulins prepared specifically against tumor substances by immunologic techniques might prove of value in treatment of cancer.

Technic. Anticancer cell antibodies were prepared against Walker 256 rat tumor by inoculating several million Walker cells into rabbits every 4 days for 3 or 4 injections, and subsequently fractionating gamma and alpha-2-globulins from their serum. Cancer cell antibody titer was then determined and if adequate, the animals were exsanguinated. As antibodies were also formed against the small amount of normal tissue not removed, these were absorbed out by modification of technic of Edwards and Ewing(3). Serum containing the antibody was kept overnight at cold temperature on an electro-magnetic stirrer, and enough of a single cell suspension of normal tissue was added to allow complete absorption of "normal" tissue antibody. The serum was then centrifuged and tested for specificity. Gamma and alpha-2-globulins were isolated by modification of Cohn's(4) "5-point variable" alcohol precipitin technic as adapted by Nitschmann *et al.*(5), and further modified to pH to correspond to animal's blood. Thirty rats were injected subcutaneously with 10,000 Walker 256 cells. Twenty were injected with antibody serum prepared as described above and 10 were controls.

Results. Immunologic studies using the agglutination technic showed that the protein fraction containing gamma and alpha-2-globulins had more than 100 times the amount of antibodies found in antiserum before it was fractionated. It was found that adsorption of normal tissue antibodies did not remove cancer cell antibodies and that adsorption technic used was effective in removing the antibodies formed against normal tissue.

In all 10 control rats, "takes" were palpable on eighth day. In 20 animals injected with antibody serum, "takes" were prevented in 12; in the remaining 8, "takes" were not demonstrable until 22nd to 25th day. The animals were observed for additional 90 days but no more "takes" were noted. It was also found that this protein fraction did not give rise to anaphylactic or toxic reactions. When the protein fraction was injected systemically into rats with previously established tumors the growths decreased in size by about one quarter but commenced to grow again after about 48 hours. The tumors in treated animals remained smaller than in controls. Direct injection of the anticancer fraction into the tumor did not appear to be more effective than its administration systemically. Injection of normal rabbit gamma and alpha-2-globulins had no effect on the tumors.

Two patients suffering from advanced cancer of the breast with supraclavicular node metastases have been treated with gamma and alpha-2-globulins isolated from sheep, which had received 6 injections of 15 million cancer cells obtained from the primary breast tumor of the patient. Three animals were used for each patient. In one patient the supraclavicular metastatic node measured 4×3 cm before injection of the antibody serum, and 24 hours after injection of the serum it measured 3×2 cm. In the other patient the supraclavicular node measured 7×4.75 cm before injection, and 24 hours after injection measured 5.6×3.75 cm. In neither case was any diminution in size noted during succeeding days. Immunologic studies showed that gamma and alpha-2-globulins had a high titer against cancer cells. In one patient a piece of cancer tissue removed after treatment did not react with the protein fraction whereas it did react prior to treatment, signifying that antibody was "coating" the cancer tissue. Following injection, one patient developed a chill, and the other, pain in lumbar region after injection, but in neither case could these reactions be considered of major significance.

Summary. We produced antibodies by injecting cancer cells obtained from Walker 256 tumor into rabbits; after several injections of the cell antigen, the rabbits were exsanguinated, and the serum fractionated. Antibodies to normal tissue were adsorbed out by expo-

sure to normal tissue. Injection of the globulin fraction prevented "takes" of Walker 256 cells in slightly over half of rats inoculated with 10,000 cells. The globulin antibody solution produced a decrease in size by about one quarter when given to rats with tumors 2 to 3 cm in diameter, but the tumor masses did not disappear. The method was tried in 2 patients with decrease in size of the tumor but not disappearance.

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Effect of Thyroidectomy on Pancreatic Amylase.* (24514)

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Hypophysectomy of the rat induced a striking reduction in weight of the pancreas (1,2) and in size and granulation of acinar cells(3). Also, amylolytic(1,4), proteolytic (5) and lipolytic (unpublished) activities were depressed significantly. That some of these changes may have resulted from deficient thyroid secretion was indicated by the occurrence after thyroidectomy of partial pancreatic atrophy(6) and reduced total lipolytic activity. Furthermore, morphological evidence of stimulation appeared following administration of thyroid substance to nonoperated rats(7-11). The purpose of our experiments was to observe the effect of thyroidectomy on pancreatic amylase. If the direction and magnitude of change were similar after both hypophysectomy and thyroidectomy, additional evidence would be provided to support the hypothesis that a significant portion of hypophyseal regulation over enzyme production by the pancreas is mediated by the thyroid gland.

Methods. Young adult female rats of Sprague-Dawley strain were used, with the following treatments given to one-half of the rats in each experiment: Exp. 1, surgical thyroparathyroidectomy; Exp. 2, parathyroidectomy by electrocautery; and Exp. 3, surgical thyroparathyroidectomy in addition to 100 μc of I^{131} subcutaneously 13 days later. Radioactive iodine was given to the third group to insure destruction of thyroid fragments which are often left after surgical thyroidectomy. Completeness of gland removal was verified by microscopic examination of the cervical region in all rats of Exp. 1 and in some of Exp. 3. Those which retained fragments of significant size were eliminated. Exp. 2 acted as a control for Exp. 1 in that an opportunity was provided to observe the effect of parathyroidectomy alone. After operation the controls were pair-fed against the operated rats. The animals in Exp. 1 and 3 were fed "Low Iodine" Test Diet (Nutritional Biochemicals Corp.). To this was added 1 g glucose/100 g of test diet for operated animals, and the same amount of glucose in addition to 3.27 mg of KI for control animals. Animals in Exp. 2 were fed powdered Purina Laboratory Chow. All rats were given water *ad lib.* but no food for 24 hours prior to termination of experiment. After killing the rat by blow on head, the pancreas was excised,

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TABLE I. The Effect of Thyroparathyroidectomy and Parathyroidectomy on the Amylolytic Activity of the Pancreas.

Treatment	No. of rats	Days postop.	Mean body wt (g) Initial	Mean body wt (g) Final	Mean pancr. wt (mg)	Mean amylase activity (mg glucose) Per unit wt†	Mean amylase activity (mg glucose) Total
Exp. 1							
Control	7		164 ± 4*	187 ± 7	889 ± 37	1.73 ± .15	31,293 ± 3470
Thy.	7	64–72	155 ± 4	167 ± 8	450 ± 20	1.02 ± .12	9,139 ± 1210
					P <.001	P <.01	P = .001
Exp. 2							
Control	9		171 ± 3	207 ± 5	947 ± 36	.85 ± .12	32,584 ± 4939
Parathy.	9	62–69	165 ± 3	216 ± 3	922 ± 21	.88 ± .08	32,697 ± 3252
					P >.5	P >.8	P >.9
Exp. 3							
Control	10		145 ± 1	173 ± 9	846 ± 48	.79 ± .04	26,979 ± 2322
Thy. + I^{131}	10	66–72	145 ± 1	148 ± 8	468 ± 23	.33 ± .02	6,058 ± 308
					P <.001	P <.001	P <.001

Thy. = Thyroparathyroidectomy; Parathy. = Parathyroidectomy.

* Stand. error of mean.

† Unit wt of pancreas in Exp. 2 and 3 = 0.025 mg; in Exp. 1, 0.05 mg.

weighed and homogenized in chilled physiological saline. The homogenate was diluted with physiological saline to a concentration of 0.1 g of pancreas/4 l of saline in Exp. 2 and 3 and to 0.1 g/2.0 l in Exp. 1, and filtered thru glass wool. To 1 ml of this homogenate was added 2 ml of 0.2 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.0) and 5 ml of 0.5% Lintner soluble starch made up in 1% NaCl, the latter warmed previously to 37°C. A similar preparation was used as a blank except that it contained 1 ml of water instead of the homogenate. Both tubes were incubated at 37°C with constant agitation. After 10 minutes incubation, the tubes were placed in boiling water bath for 5 minutes to stop enzyme activity in tube containing the homogenate. The tubes were cooled in running tap water and protein was precipitated by addition of 1 ml of 5% $ZnSO_4$ and 1 ml of 4.5% $Ba(OH)_2$ followed by centrifugation at 2000 rpm for 3 minutes. The Nelson-Somogyi method was employed for determination of amount of reducing substance released by amylase. Amylase activity was expressed as mg of glucose released from starch in 10 minutes at 37°C by 1 ml of homogenate (0.025 mg of pancreas in Exp. 2 and 3 and by 0.05 mg of pancreas in Exp. 1) by reference to a standard curve. The significance of the difference between mean values for control and experimental groups was determined by the Student-Fisher t formula.

Results. Surgical thyroparathyroidectomy significantly reduced the weight of pancreas from 889 ± standard error 37 mg for controls to 450 ± 20 mg. The amylolytic activity after thyroparathyroidectomy was reduced from 1.735 ± 0.147 mg to 1.018 ± 0.125 mg/unit weight of pancreas and from 31,293 ± 3470 mg to 9139 ± 1210 mg for the whole gland (Table I). After surgical thyroparathyroidectomy, amylase/unit weight of pancreas was reduced 41% while after thyroparathyroidectomy followed with I^{131} there was a 58% reduction (Exp. 3). Reduction in total amylase and amylase/unit weight of pancreas was significant at the 1% level or better in Exp. 1 and 3. Surgical parathyroidectomy had no effect on pancreatic weight or amylolytic activity.

Discussion. Hashimoto(11) studied the effect of thyroidectomy on pancreatic amylase in rats. Unfortunately, because of the small number of animals and failure to remove all of the thyroid gland, his conclusions carry little weight. He observed that secretion of amylase was depressed only during the brief period of 2 weeks after thyroidectomy and that between 15 and 17 days there was no evidence of an influence of thyroid deficiency on pancreatic secretion. Hashimoto concluded

that after 2 weeks some mechanism (which in his case appeared to be regeneration of thyroid tissue) had compensated for glandular deficiency. Our results show clearly that 9 to 10 weeks after thyroidectomy an approximately 50% reduction in pancreatic amylase is evident.

Since the effects of hypophysectomy and thyroidectomy on pancreatic weight and amylase are of comparable magnitude it may be inferred that a large proportion of pituitary control over pancreatic acini is exerted through the thyroid gland. However, no aspect of altered pancreatic function in the hypophysectomized rat has yet been fully restored by therapy with thyroid hormones.

Summary. After surgical thyroparathyroidectomy, with or without additional treatment with I^{131}, the pancreatic weight and amylolytic activity/unit weight and /total weight of the pancreas were reduced significantly, the fall in concentration of enzyme being greater with the latter experimental procedure.

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Toxic Protein Degradation Products. (24515)

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Vaughan(1) first noted that toxic polypeptides may be prepared from a variety of bacterial, plant and animal proteins by relatively simple chemical procedures. As more recent investigations(2-10) suggest that a number of products of endogenous proteolysis may possess substantial physiological activity it was decided to reinvestigate several of Vaughan's easily accessible and toxic "protein split products" with the aid of more modern procedures than those used previously(1) with the expectation that the results of such a study would be useful for detection and examination of similar products of *in vivo* origin. Before presenting our own observations it is necessary to refer to some results obtained by Vaughan and his associates(1). When bacterial and other proteins were treated with boiling 2% ethanolic sodium hydroxide these workers obtained, after neutralization of the reaction mixture with hydrochloric acid, ethanol-soluble fractions containing "protein split products" which, when injected into guinea pigs, progressively elicited all symptoms associated with anaphylactic shock, i.e., peripheral irritation, muscular incoordination, severe cyanosis, convulsions and death by respiratory failure. Such fractions invariably gave positive protein tests but a negative Molisch test. The preparations were hydroscopic golden-yellow powders which were readily soluble in water. Because of the slightly acid reaction of aqueous solutions of the preparations, Vaughan(1) concluded that the products were acidic in nature and that their physiological action was associated with blocking of some vital basic groups of tissue and serum proteins. Fractionation of the above materials failed to give crystalline products but did lead to preparations with con-

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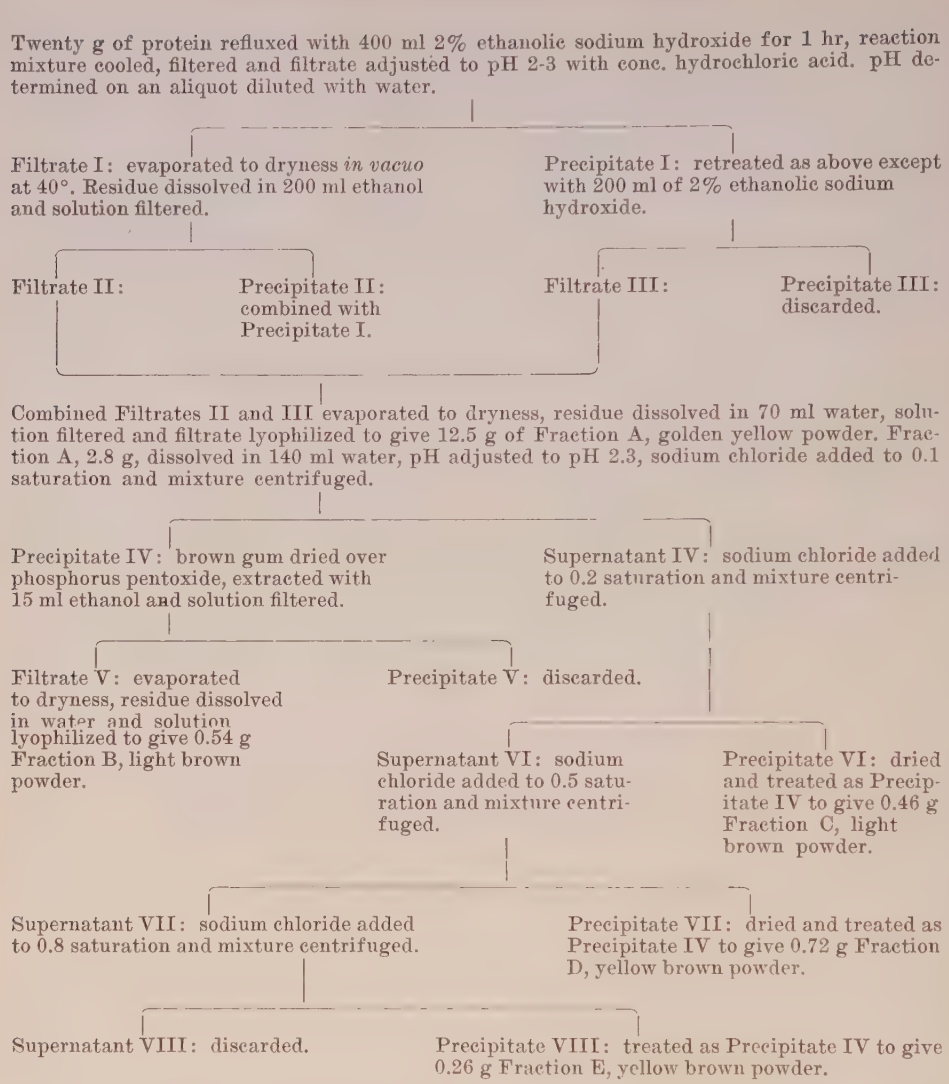

FIG. 1. Treatment of bovine serum albumin and fractionation of toxic polypeptides.

siderably enhanced toxicity. The most toxic preparations were lethal to guinea pigs when administered intravenously or intracardially in doses of approximately 1 mg/kg. However, they did not possess antigenic properties and were non-toxic when given orally.

Materials and methods. The materials employed in our study were Armour crystalline bovine serum albumin, Merck ovalbumin and edible gelatin, treated with 2% ethanolic sodium hydroxide, as described for bovine serum albumin in the flow sheet given in Fig. 1. Homogeneous reaction mixtures were obtained with bovine serum albumin and gelatin while with ovalbumin a small amount of precipitate failed to dissolve. In the toxicity tests, guinea pigs weighing approximately 500 g were employed whenever possible. However, animals deviating considerably from this weight occasionally had to be used. The animals were observed continuously for 90 minutes after injection, then checked after a lapse of 16 to 20 hours. A lethal dose usually killed in 2 to 20 minutes; death after survival for one hour was extremely rare and animals receiving a sub-lethal dose recovered within 24 hours. Toxicity tests on many intermediate fractions were performed on groups of 2

to 4 animals simply as a guide for subsequent operations. Groups of 4 to 8 animals were used for evaluation of toxicity of the final products.

Results. The unfractionated ethanol-soluble polypeptides, *i.e.*, fraction A, prepared from bovine serum albumin or ovalbumin were lethal when administered intraperitoneally at level of approximately 100 mg/kg. The same fraction prepared from gelatin was not toxic. When sodium chloride was added to aqueous solutions of fraction A and the precipitates collected successively at 0.1, 0.2, 0.5 and 0.8 saturation, the precipitates obtained at 0.5 saturation were more toxic than those obtained at lower or higher concentrations of sodium chloride (Fig. 1). Fraction D prepared from either bovine serum albumin or ovalbumin was lethal when administered at 75 mg/kg intraperitoneally or 1 mg/kg intracardially whereas the minimum lethal dose of fraction E was estimated to be approximately 90 mg/kg intraperitoneally or 5 mg/kg intracardially.

A solution of fraction D obtained from bovine serum albumin was placed in cellophane casing and dialyzed against distilled water. The dialysis was followed by determination of optical density at 280 mμ. After 24 hours it was estimated that approximately $\frac{2}{3}$ of the original material within the casing had dialyzed whereupon both the dialysate and undialyzed portion were collected and lyophilized. The undialyzed fraction when administered either intravenously or intracardially was lethal at a dose of approximately 2 mg/kg whereas the fraction which had dialyzed was lethal at a level of 0.5 mg/kg. This latter material was approximately twice as toxic as Vaughan's most potent preparation(1).

Electrophoresis of fractions A and D (Fig. 1), on filter paper (Whatman No. 1) at pH 4.1 (acetic acid-sodium acetate buffer) and 8.53 (veronal buffer) did not lead to significant separation of the various components. However, it did establish the cationic nature of these fractions and indicated the presence of many closely related species of similar electrophoretic mobility in the material under investigation. For example, when a preparation of fraction D derived from bovine serum albumin was subjected to electrophoresis at 600 volts and pH 8.53 for 4 hours a diffuse wedge with a spread of approximately 20 cm from point of application towards the cathode was observed. However, more than 50% of the material had migrated less than 6 cm from point of application. While these data, and those obtained from dialysis experiments, can only suggest that fraction D contains cationic components whose molecular weights may vary from 5,000 to 20,000, they explain why Vaughan(1) was led to the erroneous conclusion that the products of the limited alkaline degradation of proteins are acids. It is now clear that the acidic reaction exhibited by aqueous solutions of preparations such as fraction A is due to the fact that these materials are hydrochlorides of basic substances.

With the recognition of the intrinsic basic nature of fraction A, preparations of this fraction, derived from bovine serum albumin or ovalbumin, were dissolved in water and introduced into columns charged with the weakly acidic ion exchange resin IRC-50 (Rohm and Haas) which had been prewashed with an acetic acid-sodium acetate buffer of pH 4.7. Initially the charged columns were washed with water and the "neutral" effluent collected in successive 25 ml portions. The optical densities of these fractions were determined at 280 mμ and when these values were plotted against the fraction number only a single broad maximum was observed. A material balance revealed that the bulk of the original material was retained on the resin. Therefore, it was eluted with 0.1 *N* hydrochloric acid and fractions of the eluate were collected as before. Again spectrophotometric examination of the various fractions revealed only a single diffuse peak indicative of the presence of numerous components of very similar properties. Paper electrophoresis of the desalted "neutral" effluent and the hydrochloric acid eluate showed both to be basic polypeptides although the former appeared to possess fewer basic groups as indicated by its lack of retention on the column and its less intense coloration with bromphenol blue when the latter substance was applied following paper electrophoresis. The hydrochloric acid eluate was lethal at a level of 0.5 mg/kg when given

intravenously. The "neutral" eluate was approximately one-half as toxic. There was no significant difference in toxicities of the fractions derived from bovine serum albumin or ovalbumin.

Elementary analysis revealed that preparations obtained by the various procedures possessed nitrogen contents which varied from 11.0 to 13.5% and sulfur contents from 0.6 to 1.3%. The fractions which had the highest nitrogen content and a sulfur content of approximately 1% were the most toxic.

All of the polypeptide fractions were characterized by considerable absorption at 270-280 mμ, a region in which the original proteins possessed a minimum. Acid hydrolysis failed to alter the characteristics of the absorption curves of the polypeptide fractions indicating that treatment of the original proteins with boiling 2% ethanolic sodium hydroxide produced changes at the amino acid as well as at the peptide level. Because the polypeptide fractions derived from gelatin, which is deficient in the aromatic amino acids, follow the same pattern the latter amino acids hardly can be implicated in the appearance of the new chromophore. It is also evident that absorption at 270-280 mμ is in no way related to toxicity of the degradation products.

The amino acid composition of representative toxic polypeptide preparations relative to that of the parent proteins was determined by 2-dimensional paper chromatography of the respective acid hydrolysates based upon use of t-butanol-water-formic acid and phenol-water-amonia as developing agents (11). The most striking result was the absence of cystine in polypeptide hydrolysates (Fig. 2). Since hydrogen sulfide was liberated when the original reaction mixtures were acidified (Fig. 1), it is probable that most of the cystine was lost through a process involving β-elimination of hydrogen sulfide during treatment with the 2% ethanolic sodium hydroxide(12). A similar reaction appears to have occurred with respect to serine and threonine since both of these amino acids were present only in trace amounts in the polypeptide hydrolysates. It is not known whether these structural changes were in any way associated with degradation of the original protein.

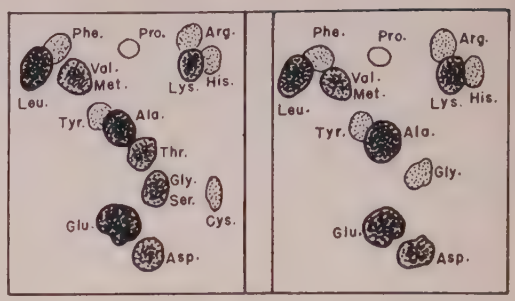

FIG. 2. Representation of paper chromatograms of acid hydrolysates of bovine serum albumin (left) and toxic polypeptide fraction derived from the same protein (right).

The remaining amino acid pattern of the polypeptide hydrolysates did not vary significantly from those of the parent proteins. It was surprising to find that arginine, which is known to give ornithine upon alkaline hydrolysis, had withstood degradation by the ethanolic sodium hydroxide reagent and there was no indication, at the amino acid level, of extensive decarboxylation of the acidic amino acids which might have been anticipated in view of the basic nature of the polypeptide preparations.

Although it is well known that the exposure of proteins to alkaline conditions may result in at least partial racemization no attempt was made to explore this area at an amino acid level largely because none of the polypeptide fractions were toxic when administered orally and those obtained from bovine serum albumin and ovalbumin were relatively non-toxic when given intraperitoneally. With this indication that the polypeptide preparations were capable of being degraded by *in vivo* proteinases and peptidases it did not appear worth while to examine their *in vitro* enzymatic degradation.

Discussion. A detailed account of the pharmacology of toxic polypeptides has been given by Vaughan(1) and by Edmunds(13). Under their influence splanchnic or sciatic nerve of dogs did not respond to stimulation, indicating peripheral paralysis of the vasomotors. Paralysis of the ganglia by large doses of nicotine failed to prevent the characteristic fall in blood pressure following injection of

the toxic polypeptides proving their action to be peripheral to the ganglia. Furthermore, it was found that epinephrine was capable of counteracting the fall in blood pressure associated with moderate doses of the peptide material thus demonstrating that the peripheral nerve endings were the structures primarily affected. However, large doses of the polypeptide appeared to have a more generalized action affecting not only the nerve endings but also the receptive and contractile substances of the blood vessels. Our own observations tend to corroborate this latter finding as pretreatment with epinephrine at a level of 0.6 mg/kg subcutaneously failed to modify the toxic action of a normally lethal dose in the guinea pig. Because of the role assigned to histamine in anaphylactic reactions it was of interest to observe that antihistaminic agents had little or no effect upon the toxic action of the polypeptide preparations. Pretreatment of guinea pigs with 2-(2-dimethylaminoethyl) (p-methoxybenzyl) amino pyridine (Neo-Antergan) at a level of 45 mg/kg intraperitoneally, which should have protected the animals against 200 lethal doses of histamine, in no way altered the toxicity of the polypeptides.† It should be noted that antihistaminic agents also were unable to prevent the inflammatory response of tissue to the polypeptides which Spector(5) isolated from turpentine induced tissue exudates.

Danielopolu and Simionescu(14) have postulated that primary shock is not due to action of histamine but to liberation of acetylcholine. However, pretreatment of guinea pigs with atropine sulfate, at levels of 10 mg/kg subcutaneously or 5 mg/kg intracardially, failed to protect animals given a lethal dose of the polypeptide. As no symptoms of toxicity were observed in the mature control animals when either Neo-Antergan or atropine sulfate were administered at the levels indicated it may be concluded that the toxic action of the polypeptides is not mediated in a primary sense by liberation of histamine or acetylcholine nor is the former substance a component of the peptide preparation.

Addition of toxic polypeptides to an isolated strip of guinea pig small intestine in Tyrode solution, to give a concentration of approximately 1:20,000, caused moderate contraction similar to that observed by Armstrong *et al.*(15) upon exposure of guinea pig uterus and jejunum to a pain producing peptide from plasma, bradykinin, hypertensin, oxytocin, etc.

Although a direct analogy between the chemical degradation of serum proteins by ethanolic sodium hydroxide and enzymatic *in vivo* proteolysis is not permissible, it is tempting to speculate on the toxicity and physiological role of polypeptides arising from elevated *in vivo* protease activity in certain disease conditions(2). The similarity of symptoms elicited by lethal doses of toxic polypeptides to those observed in anaphylactic shock is a particularly striking example. If *in vivo* proteolysis is assumed to yield products of a toxictiy comparable to that of the polypeptide materials considered in this study, 50-100 γ intravenously would suffice to produce severe pathological symptoms in adult guinea pigs. The difficulties that would be encountered in isolating and characterizing such a small amount of material from the entire circulatory system of an animal are obvious and perhaps it is for this reason that such products have so far eluded detection.

Summary. Toxic polypeptides, similar to those obtained by Vaughan(1), have been prepared by treatment of certain proteins with boiling 2% ethanolic sodium hydroxide. These substances are soluble in water and in ethanol, are capable of dialyzing through a cellophane membrane and have been shown to be a mixture of essentially basic polypeptides of similar composition. Their characteristic absorption at 270-280 mμ does not appear to be related to their toxic properties. At an amino acid level the polypeptides differ from their parent proteins in that they contain little or no cystine, serine or threonine. The most toxic preparations, obtained by a salting out procedure, followed by dialysis or by ion exchange chromatography, while non-

† Neo-Antergan at this level was lethal to immature animals weighing approximately 250-300 g. However, intracardial injection of 20 mg/kg of histamine phosphate reversed the action and animals so treated survived.

toxic when given orally were lethal to guinea pigs at a level of 0.5 mg/kg intravenously. Pretreatment of the animals with atropine, epinephrine or Neo-Antergan failed to prevent or modify the effect of a lethal dose of the polypeptide thus excluding liberation of histamine or acetylcholine as the principal cause of toxicity.

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Humoral Regulation of Erythropoiesis V. Relationship of Plasma Erythropoietine Level to Bone Marrow Activity. (24516)

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Erythropoietine has been demonstrated repeatedly in plasma of experimental animals following exposure to acute hypoxia and also in certain clinical disorders with severe chronic hypoxia(1). Rate of release of erythropoietine, its disappearance following discontinuance of the stimulus, and maintenance of plasma levels have not been extensively studied. Prentice and Mirand(2) noted a decrease in plasma level of erythropoietine following prolonged exposure to low O_2 concentrations at ambient pressure. Erslev(3) was unable to confirm these observations in anemic rabbits or those exposed to low O_2 concentrations. Since the conditions which influence plasma level of erythropoietine are important in the role of erythropoietine in clinical anemias, a further evaluation of those factors is here reported.

Methods and materials. Erythropoietine was assayed in fasted Sprague-Dawley rats as suggested by Fried *et al.*(4) and described elsewhere(5). Release of erythropoietine was stimulated by bleeding rats 2% of body weight or by exposing them to simulated altitude of approximately 23,000 feet (310 mm Hg) in decompression chamber. In studies on effect of chronic anemia in dogs, plasma was acidified (pH 5.5), heated 10 minutes at 90°C (internal temperature). The material was lyophilized and final preparation concentrated 5-fold to compensate for loss of activity due to heating(6) and dialyzed against isotonic phosphate buffer at pH 7.0. The effect of bone marrow function on plasma erythropoietine levels was studied in rats in which erythroid hypoplasia was produced by ionizing radiations. A dose of 400 r was delivered using a 2.5 MEV Van de Graaff generator at average dose rate of 50 r/minute measured with Bendix ionization chamber known to be air equivalent at that energy.

Results. It was possible to demonstrate erythropoietine in plasma of rats exposed for 2-3 hours at a simulated altitude of 23,000 feet. Plasma concentration of erythropoietine

TABLE I. Relationship of Plasma Erythropoietine Level to Duration of Exposure to Hypoxia.

	Altitude plasma*						
	Duration of exposure at altitude (hr)						
Normal plasma	3	6	12	18	24	36	48
5 ± .4†	11 ± 1.4	13 ± .5	17 ± 1.0		20 ± 2.0		
6 ± .9			22 ± 1.2		23 ± 1.5	16 ± 2	10 ± 1.3
6 ± 2.0				29 ± .9			12 ± 1.2

* Plasma obtained from donors following exposure at simulated altitude for varying periods.
† These values represent % of total inj. Fe^{59} present in circulating red cells 16 hr after intrav. inj. of Fe^{59}. Each figure represents mean value for group of 6 animals ± a single stand. error.

continued to rise until peak levels were observed after 12 to 24 hours of exposure (Table I). When length of exposure was prolonged to 48 hours, a substantial fall in plasma level of erythropoietine was noted (Table I). In experiments where a single bleeding of 2% of body weight was used as stimulus for erythropoietine release, a similar curve was noted, although peak values of erythropoietine were substantially less than those observed in altitude-exposed animals (Table II). Due to low peak values the decrease with time appeared relatively small, although in all 3 instances the values at 48 hours were less than those at 24 hours.

Chronic anemia with hematocrits of 20-35 was produced by multiple phlebotomies in dogs whose iron stores were maintained by parenteral administration of Imferon®. In those instances where the hematocrit was in the range of 20-28, significant levels of erythropoietine were demonstrable (Fig. 1). In dogs with higher hematocrits, however, erythropoietine could no longer be demonstrated.

The disappearance rate of erythropoietine was determined by obtaining samples of plasma from rats at various intervals after discontinuance of 16 hour exposure at simulated altitude of 23,000 feet. Results are presented in Fig. 2. The disappearance was curvilinear. An approximation of the half disappearance time was made by comparing the disappearance curve with values obtained using 25% and 50% dilutions of plasma obtained immediately after discontinuance of exposure. The half disappearance time estimated in this fashion was 3-5 hours.

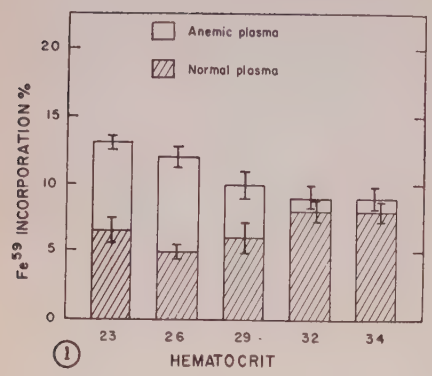
TABLE II. Relationship of Plasma Erythropoietine Level to Duration of Anemia.

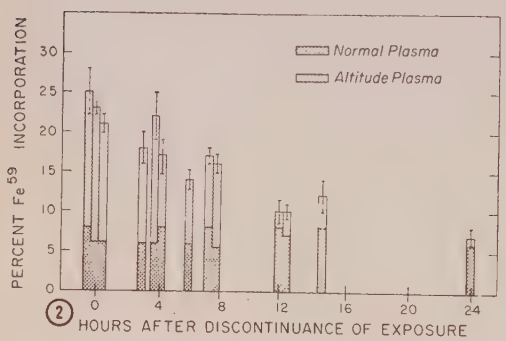
	Anemic plasma*			
Normal	Duration of anemia (hr)			
plasma	12	18	24	48
7 ± 1.7†		13 ±1	12 ± .6	10 ± .9
6 ± 1		10 ± .9	13 ± 1	9 ±1.9
10 ± 1.4	14 ± 1.7		14 ± 1.7	10 ± .9

* Plasma obtained from rats at various intervals after removal of blood equivalent to 2% of body wt.
† These values represent % of total inj. Fe^{59} present in circulating red cells 16 hr after intrav. inj. of Fe^{59}. Each figure represents mean value for group of 6 animals ± a single stand. error.

Since plasma level of erythropoietine decreased in presence of continued hypoxia, where the only apparent change was increase in amount of erythroid tissue, the relationship of bone marrow function to plasma erythropoietine was studied. Bone marrow hypoplasia was induced in rats by exposure to 400 r of ionizing radiations. At an interval of 8 hours after irradiation, the animals were exposed to simulated altitude and erythropoietine levels compared to those of simultaneously exposed non-irradiated animals. The results are given in Table III. Plasma collected from irradiated animals exposed for 48 hours increased Fe^{59} incorporation of assay animals to a level of 18-22% in contrast to the 8-12% values seen in non-irradiated altitude exposed animals.

Rate of disappearance of erythropoietine from plasma of irradiated rats when exposed to altitude was compared with that of non-irradiated altitude exposed animals. The data from these experiments are given in Fig. 3. In 2 experiments altitude-exposed animals received 400 r and in one 700 r. Clearance of erythropoietine from the plasma is somewhat slower in those animals with radiation-induced hypoplasia than in those with normal mar-





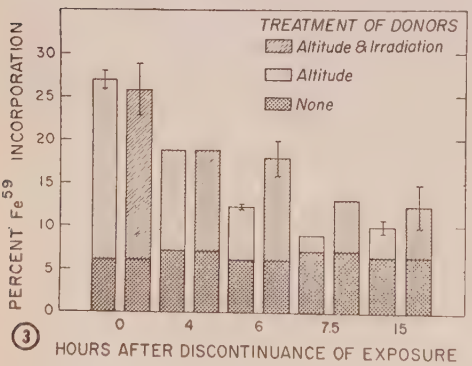


FIG. 1. Relationship of severity of anemia to plasma erythropoietine levels in the dog. Each bar represents mean 16 hr Fe^{59} incorporation of 6 animals. Small cross-bars represent a single stand. error.

FIG. 2. Disappearance of erythropoietine from plasma after discontinuance of hypoxia. Plasma was obtained from donor rats at time intervals indicated on horizontal axis, after 16 hr exposure at simulated altitude of 23,000 ft. Each bar represents mean Fe^{59} incorporation of 6 animals. Small lines or cross-bars represent a single stand. error.

FIG. 3. Disappearance of erythropoietine from plasma of irradiated and non-irradiated animals, at various time intervals following exposure to altitude. Each bar represents 1-3 experiments of 6 animals each. At those instances where more than one experiment was done the small cross-bar indicates range of mean Fe^{59} incorporations.

TABLE III. Comparison of Plasma Erythropoietine Levels in Altitude Exposed Animals with and without Radiation Induced Marrow Hypoplasia.

Normal plasma	Altitude plasma*			
	Irradiated		Non-irradiated	
	18 hr	48 hr	18 hr	48 hr
9 ± 1.2†	29 ± 1.5 29 ± 1.2 31 ± 2.7	17 ± 1.3 21 ± 1.5	30 ± 1.6	12 ± 1.1
7 ± .6	29 ± 2.3	17 ± 1.5	28 ± 1.9	8 ± 1.6
9 ± 1.2	25 ± 1.2	18 ± 2.6	28 ± 2	11 ± 1.4

* Plasma obtained from donors following exposure at simulated altitude for varying periods.
† These values represent % of total inj. Fe^{59} present in circulating red cells 16 hr after intrav. inj. of Fe^{59}. Each figure represents mean value for group of 6 animals ± a single stand. error.

rows. In all experiments average erythropoietine levels observed in irradiated animals 6-15 hours after discontinuance of exposure was greater than that of normal animals exposed to altitude.

Discussion. Data presented demonstrate that following acute exposure to hypoxia there is prompt release of erythropoietine into the plasma, peak values being achieved within 12-24 hours. In animals exposed over a longer period a fall in erythropoietine titer occurred. The data presented herein as well as that previously reported indicate that magnitude of initial response is dependent upon severity of the hypoxia.

The level at which plasma erythropoietine is maintained in chronic hypoxic states appears determined by state of bone marrow function as well as degree of hypoxia. Thus in irradiation-induced marrow hypoplasia, higher erythropoietine levels were present than in non-irradiated animals exposed to a similar degree of hypoxia. Moreover rate of removal of erythropoietine from plasma was somewhat faster in those animals with normal or hyperplastic marrows than in those with marrow hypoplasia.

The implication of these observations in considering the role of erythropoietine in clinical disorders is important. Thus one might anticipate a low plasma level of erythropoietine in mild to moderate anemias asso-

ciated with marrow hyperplasia. Conversely, high levels of erythropoietine would be expected in anemias with similar hemoglobin levels but where marrow is hypoplastic or aplastic. Such a relationship has been noted by us as well as others(1,5,7). We observed the highest erythropoietine levels in aplastic and refractory anemias, where Fe^{59} incorporation values as high as 50% have been produced in the starved assay animal. In patients with sickle cell anemia and hemoglobin levels of 7-8% with marked erythroid hyperplasia, values for Fe^{59} in assay animals were 10%.

The factor of marrow utilization could also account for the observation that erythropoietine is difficult to demonstrate in animals with phenylhydrazine induced anemia unless the hematocrit is in the range of 10-20. In Erslev's studies where erythropoietine was found in animals 48 hours after induction of anemia, hemoglobin values were less than 7(3). Similarly in our experience with chronic blood loss in dogs it was necessary to produce a substantial anemia before erythropoietine could be demonstrated. It is not readily possible to gauge degree of hypoxia produced by a given degree of anemia. However, the supposition that the graded erythropoietine response to severe degrees of anemia is mediated though production of hypoxia is compatible with previously demonstrated correlation between degrees of hypoxia and level of erythropoietine (8).

We concluded that plasma level of erythropoietine in chronic hypoxia is dependent upon severity of hypoxia together with the state of bone marrow function. Urinary excretion of erythropoietine has also been demonstrated (9,10) but the threshold for excretion remains to be determined as does the possible role of liver inactivation(2,11).

Summary. Data have been presented on rate of appearance and disappearance of erythropoietine. Plasma level of erythropoietine rises promptly in response to hypoxia but on continued exposure declines. In rats with radiation-induced hypoplasia, this fall in erythropoietine titer was modified. Accordingly, it is suggested that the marrow utilizes erythropoietine and the clinical implications are discussed.

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Difference in Biological Behavior Between Primate and Beef or Whale Pituitary Growth Hormones.* (24517)

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Isolation and characterization of pituitary growth hormones (somatotropins) derived from several mammalian species have been reported, and these hormones have been shown to manifest physical and chemical differences (1-4). It was deemed of interest to investigate the growth-promoting activity, as evidenced by body-weight gain of hypophysectomized rats, of the somatotropins from pituitary glands of these various species.

Methods. Female rats of the Long-Evans strain were used. The animals were hypophysectomized at 28 days of age and injections were begun 14 days after operation. Animals which had gained more than 5 g in the postoperative period were discarded. All rats were maintained on stock diet *ad libitum*.† The somatotropins were isolated by the published procedures from beef(5), human(6), monkey (6) and whale(3) pituitary glands. The hormone was dissolved in cold distilled water at pH 9.0, with a hormone concentration of 50 μg/ml. Fresh solutions were prepared every 5 or 7 days. The rats were injected once daily with 0.5 ml of the test solution by the intraperitoneal route. Body weights were determined to the nearest gram at intervals of from 2 to 4 days. Growth-promoting activity of each somatotropin preparation was also assayed by the tibia test(7) in hypophysectomized rats.

Results. Somatotropins isolated from human, monkey, whale, and beef pituitary glands were found to possess almost identical growth-promoting potency according to the tibia test (Table I). Results on the basis of body weight gain in hypophysectomized rats are summarized in Figs. 1 and 2. It is of interest to compare, in Fig. 1, the results obtained at 10 days with those obtained during the following 10 days. Within the first 10 days, all somatotropins tested elicited a weight gain in the experimental animals of from 11-17 g. The groups receiving whale and bovine somatotropins tended to manifest a greater gain than did the groups receiving human and monkey hormones; however, these differences were not statistically significant. By 20 days, very evident differences appeared. At this time, body weight of the groups receiving primate somatotropins had ceased to increase, whereas continuing weight increases were manifested by the groups given bovine and whale hormones. These differences between primate groups and whale-bovine groups are highly significant statistically. It is therefore evident that somatotropins of various species are not comparable on the basis of the body-weight test when the experimental period is longer than 10 days.

Also of interest was the observation that injections of both bovine and whale somatotropins, when continued at the same daily dose level (25 μg/day), were capable of inducing body-weight increases over a long experimental period. Whale and bovine groups were still gaining weight after 90 days of hormonal administration (Table II). Thus, a further distinction between the primate hormones and those of the other species may be made, in that whale or bovine somatotropin is

TABLE I. Bioassay by Tibia Test of Growth Hormone Isolated from Human, Monkey, Whale and Beef Pituitary Glands.

Species	No. of rats*	Tibia width, μ
Bovine	9	217 ± 5†
Whale	5	220 ± 4
Monkey	12	225 ± 5
Human	8	213 ± 2

* Total dose of 20 γ inj. over a period of 4 days; tibia width of control animals receiving saline averaged 155 μ.
† Mean ± stand. error.

* Supported by grants from Am. Cancer Soc., and Albert and Mary Lasker Fn.

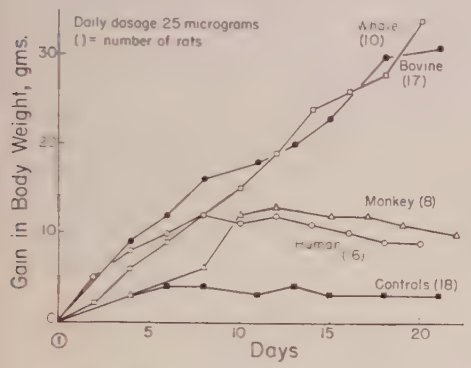
† Stock diet consists of 67.5% ground whole wheat, 15% casein, 7.5% skim milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 6.75% hydrogenated vegetable oil, 1% fish oil concentrate and 1 μg of KI/g of the diet.

TABLE II. Body Weight of Hypophysectomized Female Rats Injected with Bovine and Whale Somatotropins for 90 Days.

Group*	No. of rats	Body wt (g) Onset	40th day	60th day	Autopsy	Body wt gain in 90 days (g)
Control	10	74 ± 3†	85 ± 4	88 ± 3	92 ± 4	18
Bovine hormone	9	82 ± 3	129 ± 7	147 ± 8	167 ± 12	85
Whale "	10	83 ± 2	141 ± 5	162 ± 7	187 ± 8	104

* Daily dose, .025 mg; controls received no inj. † Mean ± stand. error.

capable of effecting continuous body-weight gain whereas human or monkey hormones exhibit a plateauing of effect after 10 days of hormonal injections. These differences are further demonstrated in Fig. 2. In the group which initially received human somatotropin, an attempt was made to reinitiate body weight gain by switching to the monkey hormone; this was without effect. Whale somatotropin, however, was capable of reinitiating body-weight gain at a rate comparable to that observed in animals that had been given whale growth hormone from the beginning (Fig. 1). Indeed, the same type of result was obtained in rats whose weight had reached a plateau with monkey somatotropin; here, although human growth hormone was without effect, bovine hormone proved very effective.



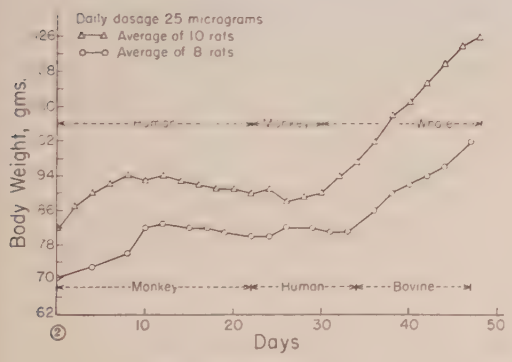


FIG. 1. Curves representing avg body-wt gain in hypophysectomized female rats for 20-22 days. Animals were hypophysectomized at 28 days of age and inj. begun 14 days later with whale, bovine, monkey and human growth hormones.

FIG. 2. Curves representing avg body-wt gain in hypophysectomized female rats for 47-48 days. Animals were hypophysectomized at 28 days of age and inj. begun 14 days later with human, monkey, bovine or whale growth hormone.

Summary. Growth hormones isolated from beef, whale, monkey and human pituitaries have been investigated with respect to their ability to elicit body-weight gain in hypophysectomized female rats. Whale and beef hormones elicited a continuous increase of body-weight for 90 days, whereas primate somatotropins exerted this effect only for 10 days. After 10 days of injections with monkey or human hormones, animals became resistant to the effect of primate somatotropins, although no corresponding decrease in response to whale or bovine hormones was evident.

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Parabiosis in Rabbits as Preliminary to Homotransplantation of Tissues. (24518)

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Homotransplantation of specialized tissues in higher animals and in man is unsuccessful because of adverse immunologic reactions that result in necrosis of the transplant. Homograft rejection is probably an antigen-antibody reaction between host tissues and transplant. Demonstration of tissue antibodies by fluorescein technics confirms this concept(1, 2). Medawar(3,4) using skin grafts and Dempster(5) working with kidney transplants have also demonstrated that homologous transplantation sensitized the host to future transplants. Secondary transplants necrotized in a shorter time than primary grafts, and tertiary transplants had even a lesser life span. Renewed interest in homotransplantation has been stimulated by the work of the Kamrins(6) and a clinical study of Merrill, Murray, Harrison and Guild(7) who reported transplantation of a kidney from one identical twin to another. This demonstrates that a kidney can function after temporary cessation of its blood supply and denervation. But transferring a kidney from one identical twin to another does not constitute true homotransplantation. The Kamrins, working with rats, reported that parabiosis was a means of overcoming immune reactions that retard homotransplantation of tissue. After litter-mate rats were maintained in parabiosis for 25 days kidney slices were exchanged between the parabiotic rats. Pieces of renal tissue survived as permanent homografts as long as the rats remained in parabiosis. Kamrin later reported that the transplanted kidney slices remained viable after separating successful parabionts as skeletonized parenchymal structures, but with a well vascularized stroma. Evidently separating the parabionts abrogated the neutralizing mechanism slowly with enough protection remaining to prevent sloughing and necrosis of the homograft. Our experiments consisted of placing young litter-mate rabbits of the same sex in parabiosis as a preliminary to homotransplantation of tissues, to repeat Kamrin's work in an animal with a more complex immunologic system. The rabbit has 4 blood types, whereas the rat has a single type.

Methods. The method of parabiotic union used was a modification of the procedure of Sauerbruck and Heyde(8,9). Forty-six rabbits were placed in parabiosis successfully. Prior to surgery the rabbits were anesthetized with intravenous sodium pentobarbital. The dose used was 0.5 cc/kg of body weight, given into ear veins. As a rabbit can be killed by overdose of pentobarbital (or any other anesthetic agent) extreme caution was used in administering the anesthetic agent. Rabbits were then placed parallel to each other on an operating board and the skin prepared for operation. Thirty of the 46 rabbits were placed in parabiosis as follows: Parallel incisions were made in adjoining mid axillary line of each animal from last rib to the inguinal area. Abdominal fascia, muscles, and peritoneal layers were then incised in line of the incision. The corresponding layers of each animal were then anastomosed with 4-0 silk sutures placed on atraumatic needles. In the remaining 16 rabbits intact peritoneal layers were placed in adjacent positions sutured to each other but not opened. Skin and muscle layers were then anastomosed as previously. With the later method it was anticipated that there would be less rapid cross circulation between the 2 animals and internal herniation of the intestines would be prevented. At termination of operations, all animals were given appropriate doses of penicillin and streptomycin for the first 3 post-operative days. None of the post-operative animals struggled to free themselves from parabiotic union. All were docile and behaved normally. In some animals, agents that modify immune processes were administered at time of opera-

tion. The majority of this group were injected with a solution of Evans Blue dye(10, 11,12). Four animals received an immunologic paralyzing dose of pneumococcous polysaccharide. Evans Blue dye (T-1824), an isomer to trypan blue (T-1835) is of low toxicity. After parenteral administration it combines mainly with serum albumin, and to a lesser degree, globulin, and so exerts a modifying effect on the immune system. The other method to obtain alteration of immune processes was parenteral injection of a polysaccharide obtained from pneumococcus according to the method of Felton(13.14.15). Felton found that intraperitoneal injection of 0.002 mg/kilo of pneumococcus polysaccharide in mice will act as antigen and stimulate creation of antibodies. However, increasing the dose to 0.5 mg/kilo created an immunologic paralysis, so that an animal thus treated was unable to develop antibodies to a later immunizing injection during most of its life span. These mice were hypersusceptible to infection. This effect can be reached by giving a single large dose or by repeated small doses as long as the total is 0.5 mg/kilo. Injected polysaccharides are fixed in the reticulo-endothelial cells and in fibroblasts throughout the body, thus exerting an effect on the animal's immune processes.

Results. In the 30 parabiotic animals with common peritoneal cavities, 7 of the parabiotic pairs did not receive medication that could modify immune processes. In 6 of these pairs one of the partners died within 24 hours after operation while its mate remained apparently unaffected. In only one of the pairs did both parabionts maintain themselves until the tenth day.

In 4 pairs with common peritoneal cavity both rabbits were injected with 5 cc of Evans Blue dye. The injection was given prior to placing the animals in parabiotic union. Of these, one pair lived 3 days, one 9 days, and one 12 days, and one 21 days before one of the parabionts died. In 2 additional pairs only one of the parabionts were injected with Evans Blue and no dye given to the partner. In both of these pairs the rabbits without the Evans Blue died within 24 hours.

In the remaining 2 pairs of parabiotic animals with common peritoneal cavity, both parabionts were injected with an immunologic paralyzing dose of pneumococcus polysaccharide. Dr. L. D. Felton generously sent the material, and it was prepared for use by Dr. M. Tremaine, Immunology, State University of N. Y. . In one pair, death of one of the rabbits occurred on 6th postoperative day because of a strangulated small bowel hernia through the peritoneal cavity connection which was made smaller than usual. The other pair survived until the twenty-first postoperative day when one of the animals died.

As survival was so short when a common peritoneal cavity method of parabiosis was used, modification of the method was done in 16 animals or 8 pairs of rabbits. Also 8 survivors were placed into parabiosis making an additional 4 pairs. No substances that effect immunity were administered to 6 of the original 8 pairs. Three pairs separated on the 14th postoperative day because of necrosis at site of operation. In the remaining 3 pairs, one of the partners in one pair died in 24 hours, in another pair in 12 days, in the 3rd pair on the 21st day.

The remaining 2 pairs of parabiotic rabbits with intact peritoneal cavities had Evans Blue dye injected into the only one of the parabionts of each set. Necrosis of the operative site and separation occurred in one pair on 7th postoperative day. In the second set the parabiont without Evans Blue died on the 7th day.

Two of the pairs that did not receive drugs and that had separated on the 14th day were then rejoined. This time separation of one pair occurred in 6 days, and one of the rabbits of the other pair died on tenth day.

The rabbits that had been placed in parabiosis on 2 occasions, then separated after 6 days were joined for the third time. Now the rabbits separated after only the third day.

Two further pairs were then made, joining an Evans Blue survivor with a non-Evans Blue survivor. One pair separated on the 7th day and the Non-Evans Blue rabbit of the second pair died on the sixth day.

Discussion. Various methods have been

tried to obviate the immune reactions that preclude homotransplantation. Of these, whole animal x-ray radiation, administration of cortisone, large particle dyes or vital donor cells(16) led to a delay in immune reactions that destroy homografted tissues. The modifying effect is probably accomplished by action of these substances on white blood cells, reticuloendothelial system, and plasma proteins, suppressing production of the host's antibodies and delaying rejection of a homograft. Egdahl and Hume(17,18) attempted to modify this reaction by artificially maintained cross transfusion between donor and host prior to renal transplantation. However, they found that blood from a donor can also sensitize a host since the subsequent kidney transplant behaved as a secondary transplant after cross transfusion. Nevertheless the cross circulation afforded protection to the transplanted kidney for the period that cross circulation was allowed to continue. The protective effect of cross circulation is similar to that demonstrated by experiments of Kamrins. However, in our experiments a prolonged self-sustained cross circulation as demonstrated by parabiosis could not be maintained in the rabbit for more than 14 days.

Our results indicate that administration of Felton's polysaccharide antigen did not afford prolonged protection from homotransplant rejection. However the antigen was used in only 2 experiments so that further work will have to be done with this agent before definite conclusions can be drawn.

Summary. 1. Prolonged parabiosis in rabbits, which have a complex and sensitive immunologic mechanism, has been unsuccessful. 2. Using a common peritoneal cavity method of parabiosis, rapid absorption of an antigen that is lethal to one of the parabiotic animals occurred in one day. 3. Administration of Evans Blue and pneumococcic polysaccharide prolonged duration of parabiotic union in rabbits by their effect on animals' immune reactions. 4. Secondary and tertiary attempts at parabiosis are rejected more rapidly than the initial parabiosis, again confirming the sensitivity theory of homograft rejection.

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A Heat-Labile Zymosan Agglutinating Factor Observed in Rabbits after Intravenous Injections of Quartz.* (24519)

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Zymosan, an insoluble carbohydrate derived from yeast(1) has remarkable immunological properties: it combines with properdin(2), with bovine conglutinin(3) and with anti-zymosan antibodies(4). Zymosan, therefore, is capable of binding different, although perhaps not entirely unrelated serum proteins. In investigations on possible role of immunological reactions in pathogenesis of silicosis (5,6), it was observed that sera of rabbits which had received several intravenous injections of quartz agglutinated zymosan, even at comparatively high dilutions. Obviously in serum of these rabbits a zymosan agglutinating factor had appeared; this factor will be referred to as quartz-factor (Q.F.), although no claim is made that a direct causal relationship exists between its appearance and administration of quartz. As this factor did not appear to be identifiable with any proteins already known to combine with zymosan, it was studied in detail and comparison was made between its reaction with zymosan and those of properdin and bovine conglutinin.

Materials and methods. Quartz dust (99% pure quartz, particle size 1-3 μ) was suspended in saline, autoclaved and injected intravenously in 10 hybrid rabbits of either sex. Twenty mg of quartz, suspended in 2 ml of saline, were injected twice weekly during 8 weeks, each rabbit receiving a total of 320 mg of quartz. One week after last injection, the rabbits were bled and their sera tested for ability to agglutinate zymosan. Ten control rabbits were treated with the same amounts of sterile saline. Fresh bovine sera containing natural conglutinin were obtained directly at the slaughter house. *Sera* were examined either immediately after clotting at room temperature or after a period (up to 4-5 months) of preservation in frozen state at –20°C, and thawing immediately before use.

For agglutination 2-fold serial dilutions of each serum were made at pH 7.4 barbital buffer containing Ca^{++} and Mg^{++}(7) starting from 1:8. 0.5 mg of zymosan, finely† suspended in 0.1 ml pH 7.4 barbital buffer, were added to 1 ml of each serum dilution and the mixture incubated overnight at different temperatures, *viz*, 2°C, 17°C and 37°C; for every experiment each reagent was brought to the desired temperature before mixing. Three different zymosans were tested: Zymosan G-6, (obtained from Dr. M. E. Scevola of Ist. Sieroterapico Milanese) had been perepared by pyridine hydrolysis(8) and was composed of comparatively large particles (4 μ average diameter); according to its behaviour with the properdin system it could be classified in category B of Pillemer *et al.*(7) inasmuch as it combined with properdin to give a suitable RP, but did not give a good R_3; Zymosan D i.s.m., obtained from the same source, had been prepared by tryptic hydrolysis had definitely smaller particles (2 μ average diameter), and was classified in category A of Pillemer *et al.*, as it made a suitable RP and R_3; particle size and immunological properties of the third sample, Zymosan 6B 14 (obtained from Dr. M. Landy, National Cancer Inst., Bethesda), were similar to those of Zymosan D i.s.m. All 3 zymosans were agglutinated by Q.F. and conglutinin, however Zymosan G-6 gave better results presumably because its definitely coarser particles required less agglutinating factor to give visible clumping: Zymosan G-6 was therefore used throughout. *Agglutination* was observed after gently inverting the tubes thrice: presence of large macroscopic clumping was classified as a +++ reaction, while agglutination giving finer agglomerates, but visible by eye, was estimated as ++; a reaction was considered

* This work was supported by grant from European Community of Coal and Steel.

† Shaking 1 hour with rapid vibrator (Microid Flask shaker, Griffin, London) gave homogeneous zymosan suspensions.

+ when only microscopic observation showed that most particles were agglutinated. The influence on agglutination of *high ionic strength or absence of divalent cations* was studied by preparing sera in 2-fold serial dilutions with ionic strength = 0.69 veronal-NaCl buffer as used by Pillemer *et al.* to elute properdin from PZ(7) or with 0.01 M trisodium-ethylenediaminotetra-acetate (Na_3-EDTA) dissolved in saline. In some experiments sera were deprived of divalent cations by treatment with ion-exchange resin(9) and dilutions then performed in 0.15 M NaCl. Zymosan agglutinating activity of *sera treated with typhoid endotoxin or with gastric mucin* was tested by adding 0.25 mg of purified typhoid endotoxin‡ or 0.5 mg of gastric mucin§ to 1 ml of 2-fold serial dilutions (starting from 1:8) of serum, incubating 30′ at 17°C and finally adding 0.5 mg zymosan to each test tube. Agglutinating activity of *sera deprived of C′4* by treatment with 0.125 N NH_4OH(10) or with 6.0×10^{-3} M hydrazine (11) was also tested. To investigate the possibility that agglutination of zymosan by Q.F. could be due to interaction between some normal serum component absorbed by zymosan and the agglutinating factor of rabbit sera, agglutinability of *zymosan pre-treated with normal serum* was studied by performing 2-fold serial dilutions with pH 7.4 barbital buffer(7) of fresh normal rabbit serum starting from 1:2; to 1 ml of each dilution. 0.5 mg of zymosan were then added, the mixture incubated 1 hour at 17°C and frequently mixed; the tubes were then centrifuged and zymosan particles washed 3 times with barbital buffer. Finally, 1 ml of 1:32 dilution of Q.F. containing rabbit serum previously shown to agglutinate zymosan strongly, was added to the sedimented zymosan particles in each test tube; particles were resuspended and mixtures incubated overnight. Pretreatment of zymosan with fresh normal serum was also made in the presence of 0.01 M EDTA, the subsequent steps performed as before. Finally all sera were tested for *conglutinin activity* on sheep red cells sensitized with bovine antibody and treated with horse complement, as described by Coombs and Coombs(12).

Results. After intravenous administration of quartz the sera of 8 out of 10 rabbits agglutinated zymosan at titres 1:128 or 1:256. Zymosan agglutinating activity could be removed by absorbing the sera once with zymosan. With some fresh sera of quartz-treated rabbits a prozone was observed, agglutination at 1:8 being absent or definitely weaker than at higher dilutions. Sera of control rabbits did not agglutinate zymosan, while 2 out of 45 untreated farm-bred rabbits had sera agglutinating zymosan to dilutions of 1:64 and 1:128 respectively; investigation showed that these sera contained zymosan agglutinating factor which behaved in all respects (see below) as the factor induced by quartz.

The incidence of zymosan agglutinating activity amongst untreated hybrid rabbits of 4 different stocks bred in different laboratories was then made; while 2 stocks had 0% positive animals, the other 2 had 41% and 50% respectively; in total the sera of 16 out 97 rabbits agglutinated zymosan at titres varying between 1:32 and 1:128. This observation prompted the possibility that latent or active infection might determine appearance of Q.F. or similar factors in "untreated" rabbits. This was suggested by the fact that, in weekly examinations, the titres of one stock with high incidence of zymosan agglutinating activity declined in some animals while agglutinating activity appeared in others in which it had previously been absent. In an effort to determine the nature of the infectious agent (or agents), 5 untreated rabbits whose sera showed higher zymosan agglutinating activity were sacrificed; necrospy showed in one case a fibrino-purulent pleuro-pericarditis due, as ascertained bacteriologically, to *Pasteurella multocida*. The possibility that the "spontaneous" appearance of zymosan agglutinating activity is due to gram-negative bacteria appears attractive in view of some characteristics of Q.F. (see below) and is presently being investigated; it is apparent that conditions other than intravenous administration

‡ Kindly prepared by Dr. A. Di Nardo, Inst. of Microbiol., Univ. Milan, from *S. typhi* 0901.

§ Crude hog gastric mucin (Armour) 2 mg of which were shown previously to absorb >90% properdin from 1 ml human serum.

of quartz may induce its production.

Normal bovine sera also agglutinated zymosan at high dilutions. This was due to a factor different from that induced in rabbits by quartz *i.e.*, normally occurring bovine conglutinin(2). The simpler way to show that bovine conglutinin and zymosan agglutinating factor induced in rabbits by quartz have different properties is to inactivate the sera; bovine conglutinin is unaffected by heating for 30′ at 56°C, and inactivated bovine serum agglutinates zymosan as well as before inactivation(11). On the contrary Q.F. sera lose entirely their ability to agglutinate zymosan after heat inactivation.

That this is not due to simple loss of active complement was supported by the fact that addition of fresh rabbit or guinea-pig serum to heated Q.F. serum, did not restore ability to agglutinate zymosan. The effect of heating at 56°C on Q.F. is reminiscent of the total loss of properdin activity from sera heated to the same temperature.

The possibility was then considered that Q.F. might react with a number of substrates already known to combine with properdin; it was found that typhoid endotoxin and hog gastric mucin abolished agglutination of zymosan by Q.F. while not affecting that due to bovine conglutinin.

The next step was to consider the possibility that properdin could interfere with combination of Q.F. with zymosan; pretreatment of zymosan with fresh normal rabbit serum in dilutions up to 1:4 (and with some sera up to 1:8) made it inagglutinable by Q.F., while no inhibition was observed by pre-treating zymosan with fresh rabbit sera in presence of Na_3EDTA or with sera deprived of C′4 by ammonia or hydrazine.

These results show that something exists in normal fresh rabbit sera which reacts with zymosan, thus rendering it inagglutinable by Q.F.; this substance may well be properdin. It appears probable that the prozone sometimes observed with fresh Q.F. containing sera is due to a like interaction; treatment of the agglutinating sera with ammonia or hydrazine, while not affecting the limit titre of agglutination of zymosan, caused prozone to disappear.

The failure of Q.F. to agglutinate zymosan after pretreating the latter with fresh normal rabbit serum is important, in that it indicates that Q.F. combines with zymosan directly and that agglutination is not the consequence of a reaction between Q.F. and some normal serum component absorbed by zymosan; should this be the case, zymosan pretreated with normal rabbit serum should be agglutinated at least as well as the untreated one.

Possible similarities or differences between reactions with zymosan, of properdin on one side and Q.F. and conglutinin were investigated. It was ascertained that, while properdin does not combine with zymosan at temperature lower than 5°C(12), Q.F. and bovine conglutinin agglutinate zymosan equally well at 2°C and at 17°C; at 37°C agglutination by Q.F. was slightly improved while that by bovine conglutinin was at least 2 titres less than at 17°C.

At ionic strength = 0.69 bovine conglutinin does not agglutinate zymosan and preformed agglutinates are promptly reversed indicating that conglutinin is being eluted from zymosan much in the same way as is properdin(7); the behaviour of Q.F. at ionic strength = 0.69 is, however, different inasmuch as it normally agglutinates zymosan and is not eluted from it.

Presence of divalent cations was necessary for the combination with zymosan of both Q.F. and bovine conglutinin, as it is for properdin(12); neither Q.F. nor bovine conglutinin, agglutinated zymosan in the presence of 0.01 M Na_3EDTA or after treatment of sera with cation-exchange resin. The zymosan agglutinating activity was restored almost to original extent by performing dilutions of resin-treated sera in Mg^{++} and Ca^{++} containing barbital buffer(7) instead of simple saline.

The above results show therefore that Q.F. behaves differently from properdin and conglutinin. This was further borne out by investigation of conglutinin activity. As expected, all bovine sera showed strong (up to dilution 1:1024) conglutination of sheep red cells treated after Method I of Coombs and Coombs(13), no such activity was found in fresh and inactivated Q.F. sera. This differentiates Q.F. not only from bovine congluti-

TABLE I. Comparison of Some Properties of Properdin (Rabbit or Human), Quartz Factor (Rabbit) and Conglutinin (Bovine).

	Properdin	Quartz factor	Conglutinin
Agglutination follows reaction with zymosan	No	Yes	Yes
Combines with zymosan at 2°C	"	"	"
At ionic strength = 0.69	"	"	No
In presence of 0.01 M EDTA	"	No	"
After heating serum at 56°C for 30′	"	"	Yes
After absorption with *Salmonella typhi* endotoxin	"	"	"
After absorption with hog gastric mucin	"	"	"
In absence of C′4	"	Yes	"
Conglutinates sensitized sheep red cells treated with horse complement	"	No	"

nin, but also from immuno-conglutinin, obtained in rabbits by intravenous injections of particulate substances such as killed gram-negative bacteria or kaolin particles covered with horse complement. Like natural bovine conglutinin, rabbit immunoconglutinins are resistant to heating at 56°C for 30′, and agglutinate zymosan as well; their possible presence(13) in "normal" rabbits may explain our observation of a zymosan agglutinating activity which resisted heating at 56°C for 30′ in 2 out of 97 untreated rabbits. In another case the titre of the agglutination was reduced by heating from 1:128 to 1:16; this suggests the possibility that in some instances Q.F. may coexist with immunoconglutinin, to which it is obviously similar. Q.F. appears entirely unrelated with Cx-reactive protein; purified Cx-reactive protein (obtained through the courtesy of Dr. H. F. Wood, Irvington House, N. Y.) did not agglutinate zymosan either alone or after addition of fresh normal rabbit serum.

Discussion. That Q. F. needs divalent cations to combine with zymosan suggests that Q.F. does not behave as an antibody, at least not as a classical antibody. The evidence summarized in Table I, shows rather that Q.F. behaves in some respects like properdin and in other respects like a conglutinin; it might be asserted that Q.F. appears to be an intermediate between properdin and conglutinin.

Unlike properdin, however, it is not present in detectable amounts in blood of every rabbit. Not much is known concerning the mechanism of production of Q.F.: it is not known what conditions, other than intravenous administration of quartz, can determine its appearance, nor whether quartz acts directly or through some intermediate process, such as superimposed infections or cellular damages connected with widespread lesions of liver and spleen produced after intravenous injections of quartz(14).

Summary. After intravenous injections of fine quartz dust, a serum factor appeared in 8 out of 10 rabbits which agglutinated zymosan to a dilution of 1:256. A similar factor was also noted in occasional uninjected rabbits. This factor differed in certain respects from classical antibodies and appeared to be different from any other hitherto described, to react with zymosan. The conditions of its reaction with zymosan have been studied; they are in some respects similar to those of properdin, and similar to those of conglutinin. In certain respects, however, the factor induced by quartz behaves differently from both properdin and conglutinin. It is not believed that this factor is specifically induced by quartz but rather due to a non-specific immune response.

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Incorporation *in vivo* of P^{32} from Condensed Phosphates. (24520)

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The reported symptomatic benefit obtained in treatment of tumor metastasis to bone with P^{32} phosphate(1,2,3) has suggested the use of other forms of phosphate. In particular, condensed phosphates such as trimetaphosphate and polymetaphosphate (previously misnamed hexametaphosphate) appeared worthy of investigation since they have been hydrolyzed by mammalian tissues(4,5). Additional favorable features of these substances are their strong binding to proteins(6,7), and relatively high molecular weights(8). Both characteristics would tend to increase body retention, thus prolonging their action. This, in addition to ease with which these condensed phosphates can be prepared from other phosphates, has prompted the present study of localization of P^{32} from these higher forms of phosphates.

Methods. Radioactive trimetaphosphate was prepared in platinum crucibles from $NaH_2P^{32}O_4 \cdot H_2O$ by the method of Jones(9). Larger quantities of a non-radioactive preparation were obtained simultaneously for analytical purposes. The final preparation was an amber-colored clear glass, not precipitable in solution with $AgNO_3$ indicating an absence of phosphate. The pH of 1% solutions was 5.5. Phosphorus content was 28%. Polymetaphosphate also was prepared from $NaH_2P^{32}O_4 \cdot H_2O$ by the method of Jones(9). This material was a colorless transparent glass which dissolved slowly in water. In solution, it did not precipitate with $AgNO_3$. One % solutions had a pH of 5.8. Phosphorus content was 26%. Both substances probably do not represent pure compounds but contain small amounts of other condensation products. According to Jones(9), 92-95% of pure metaphosphates may be prepared by these procedures. Injections of $NaH_2P^{32}O_4$, radioactive trimetaphosphate or polymetaphosphate, were given intraperitoneally to 25 g male mice for 4 successive days, and the mice were sacrificed 24 hours after last injection. When larger bone specimens were required, 5 kg male rabbits were used. No toxic reactions or discomfort were observable. Preliminary experiments established intravenous and intraperitoneal routes as being equivalent and markedly superior to oral administration. That condensed phosphates are poorly absorbed by the intestine has been noted by other workers(10). Amount of radioactivity given was approximately $3x10^5$ counts/minute for the 3 substances. Depending upon the experiment, representative samples of pooled organs of 2-5 animals were obtained, wet-ashed with HNO_3 and H_2O_2 and aliquots plated on stainless steel planchets. Radioactivity was counted with an end window counter coupled with an automatic sample changer attachment. All counts were corrected for decay. No correction for self absorption was necessary for the mass of sample taken. Standard deviation for the counts measured is $\pm$ 2 cts/min. To evaluate biological retention of these substances, radioactivity of approximately $4x10^5$

counts/minute was given in single intravenous injection. Specific activities of both metaphosphates were about 40 μc/mg. $NaH_2P^{32}O_4$ was essentially carrier free. These may be considered equivalent in view of the tracer dose actually given. Two animals from each group were sacrificed at 24, 72 and 120 hours. Representative organs were pooled, ashed, and radioactivity assays performed as above. Localization of P^{32} in soft tissue such as liver was evaluated on the subcellular level by using ultracentrifugal fractionation of Schneider and Hogeboom(11).

Results. Table I shows results of a 4-day injection experiment using radioactive sodium trimetaphosphate, sodium polymetaphosphate and $NaH_2P^{32}O_4$. It demonstrates active concentration of P^{32} from the 3 substances in bone. The *in vitro* experiments(4,5) would suggest that the metaphosphates are rapidly hydrolyzed by tissues with high phosphatase activity, and that actual localization is the same in all cases, mainly as phosphate. For concentration in bone, both metaphosphates appear superior to $NaH_2P^{32}O_4$ when multiple injections are given, the factors being 1.8 times $NaH_2P^{32}O_4$ for trimetaphosphate and 1.4 times for polymetaphosphate. In evaluating the ratio of P^{32} concentration in bone to average concentration in soft tissue, again the

TABLE I. Tissue Localization of P^{32}.

Organ	Counts/min./g	% total counts/min./g
	A. $NaH_2P^{32}O_4$	
Liver	8,221	4.1
Intestine	8,211	4.1
Kidney	7,255	3.6
Lung	5,849	2.9
Femur	25,697	12.8
Muscle	7,620	3.8
	B. Trimetaphosphate	
Liver	10,013	3.9
Intestine	10,773	4.2
Kidney	9,463	3.7
Lung	7,445	2.9
Femur	47,480	18.6
Muscle	9,844	3.9
	C. Polymetaphosphate	
Liver	4,665	2.3
Intestine	5,319	2.7
Kidney	4,309	2.2
Lung	3,431	1.7
Femur	37,043	18.6
Muscle	4,002	2.0

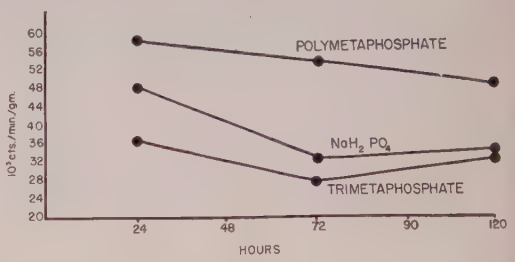

FIG. 1. Localization of P^{32} from different phosphates in bone.

metaphosphates are superior. $NaH_2P^{32}O_4$ averages 3.5, trimetaphosphate averages 5 and polymetaphosphate 8.2 times soft tissue concentration. Duplicate experiment yielded essentially the same result.

In a single injection, differential concentration of P^{32} by bone becomes more marked. After 120 hours, for $NaH_2P^{32}O_4$ it is 12, for trimetaphosphate 9.6 and polymetaphosphate 15. From rate of disappearance curve (Fig. 1), it appears that polymetaphosphate is retained longer than either metaphosphate or $NaH_2P^{32}O_4$. This is compatible with the high molecular weight of polymetaphosphate(8).

Evidence that both metaphosphates are enzymatically hydrolyzed to phosphate is obtained from radioautographs of mouse and rabbit femur and tibia. In these instances, incorporation of P^{32} into actively growing area of bone is qualitatively identical to that of phosphate.* Similarly, incorporation of P^{32} into the subcellular fractions of liver cells follows an identical pattern for all 3 phosphates (Table II). The extensive localization in nuclei and mitochondria is consistent with the high nucleic acid content of these fractions.

In view of the high alkaline phosphatase activity of certain bone tumors(12), it would appear from the present data that the metaphosphates, in particular polymetaphosphate, possess considerable therapeutic potential. These substances are being studied further.

Summary. P^{32} from trimetaphosphate and polymetaphosphate localizes preferentially in bone, particularly in actively growing areas. The metaphosphates are superior to NaH_2-

* Retention of P^{32} from the 3 phosphates in bone matrix of demineralized bone also has been found to be equivalent.

TABLE II. Subcellular Localization of P^{32} in Liver.

Fraction	Counts/min./Mg N	% total counts
	A. $NaH_2P^{32}O_4$	
Cell debris I	753	12.8
II	1,102	18.7
Nuclei	1,465	24.9
Mitochondria	1,554	26.4
Supernatant	1,011	17.2
	B. Trimetaphosphate	
Cell debris I	811	13.9
II	652	11.2
Nuclei	1,519	26.1
Mitochondria	1,618	27.7
Supernatant	1,229	21.1
	C. Polymetaphosphate	
Cell debris I	409	14.4
II	365	12.8
Nuclei	750	26.4
Mitochondria	765	26.9
Supernatant	551	19.4

$P^{32}O_4$ as a P^{32} donor to bone, the extent depending upon experimental conditions. In particular, polymetaphosphate has a longer body retention and a higher bone-soft tissue ratio. The equivalent localization of P^{32} from $NaH_2P^{32}O_4$, trimetaphosphate and polymetaphosphate into the nuclei and mitochondria of liver cells is offered as evidence that the metaphosphates are enzymatically broken down to phosphates before incorporation.

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Electrophoretic Studies of Bovine Serum. II. Concurrent Hypoglobulinemia and Natural Infections of Eperythrozoonosis.* (24521)

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Although data have been accumulated on pathogenesis of eperythrozoonosis together with descriptions of the causative agent (1-4), no work, thus far, has been directed toward physicochemical characterization of alterations manifested in the host. During the present investigations, in which bovine anaplasmosis was studied primarily, it was occasionally observed that stained blood smears showed microscopic evidence of eperythrozoonosis prior to experimental infection with anaplasmosis. This report presents results of serum protein changes concurrent with natural infections of eperythrozoonosis in calves.

Materials and methods. Cattle: In our investigation a total of 30 dairy calves of mixed breeds, ranging in age from 3 weeks to 6 months, were studied. They were housed and fed as previously described(5). Of this num-

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TABLE I. Electrophoretic Analyses of Serums from Splenectomized Dairy Calves Naturally Infected with Eperythrozoonosis after Convalescence from Surgery.

Days (bleeding intervals)	A/G*	GG	TG	TSP	EP
0	.48	2.64	5.55	8.23	—
3	.53	2.12	4.61	7.03	—
7	.45	2.18	5.02	7.27	—
10	.57	1.97	4.43	6.95	—
17	.53	2.22	5.08	7.76	—
24	.58	2.05	4.53	7.16	—
29	.77	2.27	4.26	7.52	—
31	.59	1.55	4.36	6.95	—
36	ND	ND	ND	ND	+
38	.75	1.46	3.61	6.33	+
49	.97	.62	2.94	5.78	+
56	ND	ND	ND	ND	+
60	.77	.70	3.43	6.08	—
63	ND	ND	ND	ND	+
66	.72	.89	3.62	6.21	—
70	ND	ND	ND	ND	+
73	.96	.80	3.22	6.31	—
80	.80	1.12	3.57	6.42	—
87	.64	1.91	4.29	7.03	—
94	.60	2.06	4.37	6.97	—
100	.49	2.44	5.30	7.91	—
115	.61	2.87	4.84	7.80	—
122	.57	2.65	4.64	7.29	—

* A/G = albumin/globulin ratio.

GG = gamma globulin
TG = total globulins
TSP = total serum protein
} as g/100 ml serum.

EP = appearance of eperythrozoonosis on stained blood smears.

ND = not done.

ber 17 animals, which had been splenectomized, and 3 others which were intact, showed evidence of eperythrozoonosis. The remaining 10 calves, 4 of which were splenectomized and 6 which were intact did not develop eperythrozoonosis. Splenectomy was performed to increase susceptibility to experimental infections of anaplasmosis(6). Results shown on alterations in serum proteins in all splenectomized calves are for the period after recovery from surgery. It has been reported that the serum proteins return to normal levels after several weeks convalescence (5). *Serum proteins:* Sera were separated by paper electrophoresis in barbital buffer, pH 8.6, 0.075 ionic strength. The paper strips were subsequently dyed with bromphenol blue(7) and scanned with a commercial densitometer-integrator for relative percentages of serum protein fractions. Absolute protein values on each serum fraction were determined after protein nitrogen determinations of whole serum were made using a semi-micro nesslerization technic.

Results. Since no satisfactory immunologic or serologic test was available for study of eperythrozoonosis with which results might be related to antibody, or to a degree of immunity or susceptibility, the pertinent information derived from a serum protein analysis was an indirect evaluation of the disease process. The conventional methods commonly used have considered changes in gamma globulins (GG), total globulins (TG), and total serum proteins (TSP) based on g/100 ml of serum. To a lesser extent the albumin/globulin (A/G) ratio, based on relative concentrations of these fractions, varies widely even in normal animals and does not indicate a true picture of serum protein changes that occur. A low A/G ratio with a high TSP may approximate the same figures as a high A/G ratio and a lower TSP.

After convalescence from splenectomy, as evidenced by return of normal pre-surgical serum protein levels(5) in 17 calves, severe

TABLE II. Electrophoretic Analyses of Serums from Non-splenectomized Dairy Calves Naturally Infected with Eperythrozoonosis.

Days (bleeding intervals)	A/G*	GG	TG	TSP	EP
0	.39	2.28	4.84	6.72	—
3	.48	2.05	4.62	6.84	—
7	.41	2.29	4.87	6.88	—
10	.34	2.34	4.93	6.61	—
17	.34	1.41	4.22	5.65	—
24	.46	1.80	4.42	6.44	—
29	.76	1.83	3.84	6.74	+
31	.44	1.92	4.72	6.80	—
38	.81	.72	3.23	5.84	+
49	.90	.43	2.95	5.59	+
60	ND	ND	ND	ND	+
66	.81	1.29	3.41	6.16	+
73	.61	1.64	4.13	6.65	—
80	.59	2.47	4.27	6.80	—
87	.53	2.35	4.47	6.84	—
94	.58	2.54	4.28	6.78	—
100	.54	2.37	5.21	8.01	—
115	.67	2.18	4.16	6.95	—
121	.73	1.81	3.67	6.33	+

* A/G = albumin/globulin ratio.

GG = gamma globulin
TG = total globulins
TSP = total serum protein
} as g/100 ml serum.

EP = appearance of eperythrozoonosis on stained blood smears.

ND = not done.

decreases in amounts of globulins were observed when infections of eperythrozoonosis became established. The A/G ratio increased at this time. In many cases the decreases in weights of globulins amounted to less than ¼ of previous normal values at a time when eperythrozoonosis was not detected in erythrocytes (Table I). The results obtained from 3 non-splenectomized calves also showing eperythrozoonosis were strikingly similar (Table II) in that globulins decreased, while A/G ratio increased as soon as the organism appeared in the blood.

Levels of serum proteins for 4 uninfected splenectomized calves and 6 uninfected intact calves are shown in Tables III and IV. These animals did not show decreases in globulins and increases in A/G ratio as were observed in animals naturally infected with eperythrozoonosis. The protein values were considered to be within normal range(5).

Discussion. The outstanding fact that was immediately evident was the marked decrease in serum globulins, especially gamma globulin, in calves infected with eperythrozoonosis under natural management. These changes

TABLE III. Electrophoretic Analyses of Serums from Uninfected Splenectomized Dairy Calves after Convalescence from Surgery.

Days (bleeding intervals)	A/G*	GG	TG	TSP	EP
0	.56	2.44	5.04	7.84	—
3	.61	2.16	4.89	7.86	—
7	.62	2.11	4.72	7.63	—
10	.47	2.32	4.28	6.27	—
17	.46	2.01	5.03	7.35	—
24	.57	2.29	4.84	7.59	—
31	.49	2.20	5.12	7.63	—
38	.52	2.03	4.75	7.23	—
47	.57	2.47	4.52	7.11	—
49	.39	2.49	4.49	6.25	—
59	.42	2.46	4.93	7.01	—
66	.55	1.85	4.00	6.19	—
72	.55	1.94	4.64	7.20	—
79	.52	2.00	4.76	7.23	—
86	.46	2.03	4.85	7.08	—
92	.56	1.99	4.79	7.48	—
100	.60	2.50	4.81	7.38	—
114	.54	2.06	4.17	6.40	—
119	.57	2.04	4.33	6.78	—

* A/G = albumin/globulin ratio.

GG = gamma globulin
TG = total globulins
TSP = total serum protein
} as g/100 ml serum.

EP = appearance of eperythrozoonosis on stained blood smears.

TABLE IV. Electrophoretic Analyses of Serums from Uninfected Intact Dairy Calves.

Days (bleeding intervals)	A/G*	GG	TG	TSP	EP
0	.55	1.89	4.59	7.09	—
7	.56	1.95	5.18	8.10	—
10	.63	1.97	4.45	7.27	—
17	.56	1.99	4.79	7.48	—
21	.62	1.93	4.48	7.26	—
28	.64	1.85	4.55	7.44	—
31	.61	1.96	4.26	6.84	—
38	.62	2.02	4.40	7.12	—
47	.55	2.04	4.30	6.65	—
49	.43	2.01	4.45	6.38	—
60	.48	2.05	4.62	6.84	—
65	.46	1.99	4.14	6.03	—
72	.54	2.18	4.43	6.80	—
80	.61	2.09	4.22	6.78	—
87	.64	1.94	4.15	6.82	—
100	.59	2.22	4.17	6.63	—

* A/G = albumin/globulin ratio.

GG = gamma globulin
TG = total globulins
TSP = total serum protein
} as g/100 ml serum.

EP = appearance of eperythrozoonosis on stained blood smears.

are contrary to popular schools of thought concerning alterations produced in serum proteins during infectious disease processes. It is common to recognize increases in globulins in bacterial diseases(8,9) but little or no deviation from the normal in most virus diseases (10,11) is anticipated.

Hypogammaglobulinemia has been reported in humans(12,13). In one case, where there was an almost complete absence of gamma globulin, TSP values were also low(13). Surprisingly, however, the patient had not become ill with any serious disease until she was 20 years of age. In another report(12) several patients, showing recurrent severe bacterial infections, exhibited an almost complete lack of serum gamma globulin, but in these instances TSP concentrations were normal.

Eperythrozoonosis has been reported to be an aggravating factor when coexisting with other diseases(3). In these instances the marked decrease in absolute weights of globulins, which contain protective substances and antibodies, may place the animal in a position of stress making it more susceptible to secondary infections.

The direct effect of eperythrozoonosis on globulin synthesis, the use of globulin changes

as possible diagnostic criteria for eperythrozoonosis and studies of the effects of this disease on production of antibody to other infectious agents are being investigated.

Summary. Results on serum protein changes in natural infections of eperythrozoonosis in calves are reported. Concurrent with microscopic evidence of infection in the blood, serum globulins decreased to an abnormally low level. This observation offers possibilities for diagnostic and clinical criteria. The implications of hypoglobulinemia in eperythrozoonosis are discussed.

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Successful Skin Homografts in Mature Non-littermate Rats Treated with Fractions Containing Alpha-globulins.* (24522)

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Skin homografts are always rejected when exchanged between normal immunologically-mature non-littermates of non-inbred rats (See review by Medawar(1)). Recently, Axelrod *et al.*(2) and Fisher *et al.*(3) reported that pyridoxine-deficient Wistar and Long-Evans strain rats have retained skin homografts for over 70 days. Reporting on their non-vitamin deficient Wistar controls, these authors found 24% retaining viable skin homografts for 60 days. Referring to the common finding that no experimental treatment of normal animals has resulted in permanently retained succesful homografts, Medawar(1) concluded that in the immunologically immature animals only the nucleated cell can bring about "immunological or adaptive tolerance." Woodruff and Simpson(4) working with Wistar rats noted that nucleated cells injected into animals less than 2 weeks old can induce some degree of tolerance to subsequent skin grafts. Puza and Gombos(5) demonstrated that young dogs (11 days post-partum) could be induced, by means of repeated exchange transfusions, to accept reciprocal homografts. Successful parabiosis involves reciprocal tolerance of skin, blood and muscle tissues. A small percentage of non-inbred littermates will go into successful parabiosis; non-littermates will not unite successfully. However, Kamrin(6) achieved successful parabiotic union of non-littermate rats (pen-bred Wistar strain) by injection of alpha-globulins derived from pooled rat blood serum. The blood serum was obtained by exsanguination of ageing rats from the same colony. Enhancement of non-littermate parabiosis by treatment with alpha-globulins suggested that treatment of single animals might induce tolerance to skin homografts.

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Methods. Most alpha-globulins utilized

TABLE I. Electrophoretic Analysis of Experimental Fractions.*

Serum proteins	Source	Albumin	α_1	α_2	$\alpha_1\alpha_2$	β	γ
		%					
Fraction VI	Rat		17.0	83.0			
III	"			17.0		66.8	16.2
IV-4	Human		59.5	33.9		6.6	
Whole serum (lyophilized)	Rat	63.5	4.0	4.5		22.0	6.0
Fraction IV-3† (non-lipoproteins)	Human				92.1	7.9	
Fraction V	Rat	47.6	27.0	25.4			
IV-1‡ (lipoproteins)	Human		36.9	39.2		23.9	
Fraction IV	Rat				85.0	15.0	
Miles' P. F.§	Guinea pig	17.1	53.9	10.6		18.4	
Fraction II	Rat					4.7	95.3

* All fractions examined by Antweiler Microelectrophoresis apparatus using veronal buffer at pH 8.6, ionic strength 0.10 of approximately 2 g/% samples.

† Fraction IV-3 consists of non-lipoprotein remainder after removal of lipoproteins from Human Fraction IV-4.

‡ Fraction IV-1 consists of lipoproteins found in alpha- and beta-globulins.

§ P F.—Permeability Factor described by Miles(10 a, b) and was kindly donated by Dr. A. A. Miles.

were obtained by fractionation of pooled rat blood serum (same colony) by several modifications of Cohn's method 10(6-9). Repeated analyses of components of each fraction were made with the Antweiler Microelectrophoresis apparatus using veronal buffer at pH 8.6, ionic strength 0.10. In addition, blood serum fractions were used (Table I) which contained alpha-globulins from other sources—(Human Fraction IV-4, Squibb; Fractions IV-1 and IV-3, obtained by sub-fractionation of Human IV-4; and Miles'(10 a,b) guinea-pig Permeability Factor). Lyophilized materials obtained by fractionation of rat serum were weighed and made up in 0.15 M NaCl to concentrations of 3 to 20 mg%. To each ml containing dissolved material, 0.1 ml of procaine penicillin (300,000 units/ml) was added. Control experiments using only injected penicillin showed that the antibiotic had no effect upon retention of skin homografts. Intraperitoneal injections of various serum proteins were made immediately following skin homografting and subsequently every third day, total of 5 injections. Homografts (average size 3 cm X 2 cm) from the abdomen of 20-46 day old non-littermates were orthotopically exchanged. Pressure bandages of gauze sponges and adhesive plaster, were placed over closely approximated and sutured skin homografts. Bandages were removed on fifth or sixth day. The cotton sutures were not removed.

Results. Post-operative retention of skin homografts in the control series is fairly uniform; *i.e.,* complete sloughing occurs between 17th and 22nd day (Table II). Failure of homograft is indicated by initial drying or loss of epithelium and subsequent sloughing of the hardened graft. Successful skin homografts in the experimental series are those remaining longer than 60 days. These successful homografts are marked by early union of the periphery of graft with host. The graft remains soft, pliant and of normal color; hair growth begins on clipped or spontaneously depilated surface between 7th and 12th post-operative day. Although no accurate measurements have been made of the increase in graft size, it seems that growth of homograft is somewhat slower than that of the recipient's skin. Study of union between host and successful graft by intravenous injection of trypan blue shows that the dye was taken up uniformly by both host and graft tissues. This implies that there is comparable vascularity in both. When animals bearing hardening unsuccessful homografts are injected with trypan blue, the grafts slowly become intensely colored by the dye. This finding is similar to that seen when the dye accumulates in necrotic tissue.

The best success with homografts was obtained by use of rat Fraction VI (Table II). These homografts bore hair, grew actively, and appeared identical with adjacent recipi-

TABLE II. Results Obtained by Intraperitoneal Injection of Serum Proteins.

Serum fraction	Range of dosage (total mg)	No. animals	No. failures	No. successes (to 60 days)	% success
Controls					
Homografts		20	20	0	.0
Autografts		28		28	100.0
Experimental					
Fraction VI (rat)	18– 30	12	7	5	41.5
III (rat)	35	6	4	2	33.3
IV-4 (human)	50– 75	19	13	6	31.5
Whole serum (rat)	50– 75	18	14	4	22.2
Fraction IV-3 (human)	50	10	8	2	20.0
V (rat)	50	11	9	2	18.1
IV-1 (human)	15– 20	10	9	1	10.0
IV (rat)*	44–100	84	79	5	6.0
Miles' P. F.† (guinea pig)	14– 18	12	12	0	.0
Fraction II (rat)	50– 75	14	14	0	.0

* In this large group, some failing grafts were retained for 44 days before sloughing.
† Use of this serum fraction resulted in retention of some skin homografts for 55 days.

ent skin. The electrophoretic pattern (Table I) of this rat serum fraction showed it contained only alpha$_1$- and alpha$_2$-globulins. The oldest successful homograft in this group is over 100 days and has been challenged by an additional graft from the original donor. The first graft remained healthy, the second graft remained healthy, contracted but continued to produce hair. The next best results were obtained with rat Fraction III which contains some alpha$_2$-globulins, gamma-globulins and a preponderance of beta-globulins. The 2 successful homografts reported in Table II broke down at 66 and 73 days. However, an additional group of 12 non-littermate rats treated with Fraction III shows 3 animals with excellent homografts at 56 post-operative days. Inability to obtain consistent results with this fraction may possibly be explained by the fact that, after lyophilization, Fraction III does not readily go into solution (0.15 M NaCl), but forms a thick suspension. Human Fraction IV-4 which ranks third in capability of maintaining successful skin homografts, contains mostly alpha$_1$-globulins, less alpha$_2$-globulins and beta-globulins. Although 3 of these homografts are over 100 post-operative days old, hair growth of grafts is not as thick as it was initially or as thick as in animals treated with rat Fraction VI. Fraction IV-4 was then further fractionated into Fraction IV-1 (lipoproteins) and Fraction IV-3 (non-lipoproteins) both of which contained alpha- and beta-globulins. Both of these fractions showed poor enhancing capability as compared with Fraction IV-4. All other fractions utilized in this experiment showed minor or no enhancing ability.

In our experiments with 196 animals, it was possible to test only a small spectrum of the possible dosages and times of administration. It is not known at the present time whether the dosage level used with each fraction was optimal.

Summary. This report demonstrates that tolerance to skin homografts can be induced in immunologically mature albino rats by use of serum fractions containing alpha-globulins. This finding suggests that extra-cellular substances (serum proteins) can also induce intolerance.

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Effect of Thyromimetic Agents on Oxygen Uptake of Rat Heart and Liver.* (24523)

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Barker and Lewis(1) and Barker(2) reported results which indicated that heart slices obtained from thyroidectomized rats treated with L-triiodothyronine or triiodothyroacetic acid elicited an earlier and more dramatic increase in the Qo_2 value than did other organ tissue slices. It is conceivable, as Boyd and Oliver(3) pointed out, that although certain thyroidal agents might not elicit a rise in total body oxygen consumption, they might exert a significant effect on the heart. The danger of administering such a compound to patients with past histories of coronary disease is readily apparent. If the results of Barker and Lewis and Barker could be confirmed, the method would serve as a means of ascertaining to what degree a thyroidal agent will stimulate the heart in reference to other organs. With these thoughts in mind, experiments were performed to determine the relationship of the effects of sodium L-3:5:3′-triiodothyronine (triiodo), 3:5:3′-triiodothyroacetic acid (triac) and thyroxamine on total body oxygen consumption and oxygen uptake of heart and liver slices of intact and thyroidectomized rats.

Methods. Total body oxygen consumption of fasted young adult intact rats was determined(4) prior to treatment. With thyroidectomized rats degree of induced hypothyroidism was determined at least one month following surgery. Rats with an oxygen consumption less than 20% of the simultaneously determined intact control values were considered hypothyroid. The intact and hypothyroid rats were then injected subcutaneously 4 days with either triiodo (0.5 mg/kg/day) or triac (2 mg/kg/day). An additional group of hypothyroid rats was injected 4 days with thyroxamine† (4 mg/kg/day). Fasted total body oxygen consumption was again determined on the fourth day prior to receiving the last injection of drug. On the fifth day the animals were sacrificed by stunning and exsanguination. Heart and liver slices were prepared by the use of a Stadie-Riggs tissue slicer. Oxygen uptake was determined in duplicate by direct method of Warburg using Krebs-Ringer phosphate fortified with 100 mg% glucose at 37°C with oxygen as the gas phase.

Results. In intact rats both triiodo and triac significantly ($P<0.01$) increased total body oxygen consumption and Qo_2 values for heart and liver (Table I). However, there was no significant difference in degree of response elicited by heart and liver in either the controls or in the triiodo or triac treated group. These data indicated that the heart did not respond more dramatically than the liver as proposed by Barker and Lewis(1) and Barker(2).

Thyroidectomized controls exhibited a significantly lower total body oxygen consump-

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† Obtained from Glaxo Labs., Ltd.

TABLE I. Effect of Various Thyromimetics on Total Body O_2 Consumption and Qo_2 of Heart and Liver Slices.

Group	mg/kg /day	Total body O_2 consumption, L/SqM/hr (Day 4)	Qo_2/mg wet wt (Day 5) Heart	Liver
Intact controls		7.6 ± .6*(9)†	1.11 ± .29 (8)	1.18 ± .08 (9)
" + triiodo‡	.5	10.9 ± .1 (11)	1.83 ± .47 (11)	1.80 ± .31 (11)
" + triac§	2	10.0 ± 1.0 (10)	2.01 ± .65 (10)	1.82 ± .27 (10)
Thyroidectomized controls		5.0 ± .4 (14)	1.12 ± .19 (12)	1.10 ± .14 (14)
" + triiodo	.5	9.0 ± 1.1 (8)	1.95 ± .33 (9)	1.95 ± .31 (9)
" + triac	2	8.4 ± .8 (9)	2.06 ± .41 (9)	1.86 ± .36 (9)
" + thyroxamine	4	5.5 ± .9 (7)	1.65 ± .12 (6)	1.25 ± .14 (8)

* Mean ± S.D. † No. of animals. ‡ L-3:5:3′-triiodothyronine. § 3:5:3′-triiodothyroacetic acid.

tion ($P<.001$); however Qo_2 values of heart and liver were not significantly decreased. These results are in disagreement with those presented by others(5-8).

A significant increase in total body oxygen consumption ($P<.01$) and Qo_2 values of heart and liver ($P<.01$) was obtained in thyroidectomized rats as a result of triiodo and triac treatment. Again there was no significant difference in the degree of response of heart and liver.

Different results were obtained with thyroxamine. Thyroidectomized rats treated with thyroxamine did not show an increase in total body oxygen consumption or Qo_2 of liver. There was, however, a significant increase ($P<.001$) in the Qo_2 value of the heart.

Failure of thyroxamine to elevate the total body oxygen consumption is in agreement with previous reports that it possesses little or no effect in this respect(9-12). Although relatively ineffective in increasing total body oxygen consumption, thyroxamine has been shown to elicit a sensitizing effect on the action of epinephrine on isolated smooth muscle (13,14). It is possible that the increase in the Qo_2 value of the heart as a result of thyroxamine treatment was caused by sensitizing this organ to endogenous epinephrine. It is also possible that the ability of thyroxamine to stimulate the heart while not affecting the liver or total body oxygen consumption may be a reflection of the heart's greater sensitivity to the drug. The effects seen with thyroxamine may be governed by dose and duration of administration. Large doses given over a short period of time may affect only the Qo_2 of the heart; whereas the same dose administered over a longer period may elicit an effect on total body oxygen consumption as well as on the Qo_2 of the heart.

Effects similar to those exhibited by thyroxamine may also be exhibited by other thyroidal agents at doses which do not result in an elevation of total body oxygen consumption. This theory is currently being investigated.

Summary. L-3:5:3′-triiodothyronine and 3:5:3′-triiodothyroacetic acid significantly increased total body oxygen consumption of intact and thyroidectomized rats. L-3:5:3′-triiodothyronine and triiodothyroacetic acid also significantly increased *in vitro* oxygen uptake of heart and liver slices; however, the heart did not respond to a greater degree than the liver. Thyroxamine failed to increase total body oxygen consumption or Qo_2 of liver; however, the Qo_2 value of the heart was significantly increased.

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Thymolytic Activity of 14α-Hydroxycortisol.* (24524)

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This communication reports thymolytic activity of 14α-hydroxycortisol by various methods in rats and mice and demonstrates that relative potency of this steroid compares to cortisol varies significantly with the thymolytic method employed.

Methods and design. Mouse thymolytic test A (Subcut. inj.). C-57 Brown female mice were obtained from Rockland Farms and bilaterally adrenalectomized and ovariectomized under ether anaesthesia at 24 days of age. The steroids were injected subcutaneously in 0.1 ml aqueous suspension in morning and afternoon one day after surgery and mice were autopsied 24 hours after first injection. Body weights and thymus weights were determined and results expressed as ratio of mg of thymus weights to grams of body weights. *Mouse thymolytic test B (Subcut. inj.).* In this test the steroids were injected once on day of operation and again on following day, all other conditions of the assay being the same as test A. *Rat thymolytic test C (Subcut. inj.).* Albino rats 25 days of age were bilaterally adrenalectomized under ether anaesthesia and injected subcutaneously once on day of surgery with 0.1 ml of Tween suspension of the steroid and again the following day. One day after last injection, the rats were autopsied, at which time thymus and body weights were determined. This injection schedule was the same as that of Mouse Test B. *Rat thymolytic test D (oral).* This test consisted in mixing the steroid with ground Purina Chow and feeding the mixture for 48 hours before determining body and thymus weights.

Results. The comparative thymolytic responses to graded doses of cortisol and 14α-hydroxycortisol using the adrenalectomized-ovariectomized mouse are presented in Table I. The statistical calculations (Table IV) demonstrate that tests A and B resulted in similar slopes, but that the index of precision was more favorable for test B and that a significantly lower potency ratio was found for the 14α-hydroxy derivative compared to cortisol. Even lower potency ratios were

TABLE I. Thymolytic Activity of 14α-Hydroxycortisol Compared to Cortisol in Adrenalectomized-Ovariectomized Mice.

Test No.	Steroid	Total dose, mg	No. of mice	Mean thymus ratio ± S.E.
A	0	0	39	3.91 ± .17
	Cortisol	.05	37	3.07 ± .12
		.1	38	3.09 ± .13
		.2	39	2.83 ± .13
		.4	38	1.87 ± .10
	14α-Hydroxycortisol	.05	38	3.06 ± .13
		.1	36	3.09 ± .14
		.2	36	2.55 ± .12
		.4	37	1.94 ± .11
B	0	0	18	4.54 ± .26
	Cortisol	.2	19	2.42 ± .13
		.4	20	1.24 ± .09
		.8	21	.81 ± .09
		1.6	21	.58 ± .04
	14α-Hydroxycortisol	.2	19	2.57 ± .19
		.4	21	2.05 ± .19
		.8	20	1.39 ± .14
		1.6	18	.75 ± .07

* Supported in part by research grant from N.I.H., U.S.P.H.S.

TABLE II. Thymolytic Activity of 14α-Hydroxycortisol Compared to Cortisol in Adrenalectomized Rat (Test C).

Steroid	Total dose, mg	No. of mice	Mean thymus ratio ± S.E.
0	0	26	4.03 ± .15
Cortisol	.1	19	3.52 ± .13
	.2	19	3.32 ± .20
	.4	18	2.98 ± .11
	.8	18	1.98 ± .12
14α-Hydroxycortisol	.2	12	4.03 ± .19
	.4	17	3.70 ± .16
	.8	15	3.71 ± .18
	1.6	5	3.29 ± .19
	3.2	5	3.22 ± .29

TABLE III. Thymolytic Activity of 14α-Hydroxycortisol Acetate Compared to Cortisol Acetate in Adrenalectomized Rat (Test D).

Steroid	Oral dose, mg/100 g food	No. of rats	Mean thymus ratio ± S.E.
0	0	10	3.55 ± .12
Cortisol acetate	2.5	10	2.67 ± .23
	5.	9	2.00 ± .19
	10.	8	1.06 ± .19
14α-Hydroxycortisol acetate	5.	9	3.34 ± .13
	10.	9	2.95 ± .12
	20.	9	2.73 ± .24
	40.	9	1.13 ± .22

TABLE IV. Thymolytic Activity of 14α-Hydroxycortisol as Compared to Cortisol in Rat and Mouse. (Calculations by Methods of Bülbring(1) and Fischer(2).)

Test #	Species	Steroid (No. of animals)	Standard (No. of animals)	Combined slope, b_c	Index of precision, λ	Relative potency, % ± S.E.
A	Mouse	14α-Hydroxycortisol (109)	Cortisol (115)	−1.97	.37	109 ± 13
B	"	" (78)	" (81)	−2.00	.26	60 ± 6
C	Rat	" (42)	" (74)	−1.30	.47	12 ± 3
D	"	14α-Hydroxycortisol acetate (36)	Cortisol acetate (27)	−2.40	.24	19 ± 3

found when rats were used. In an assay (test C) involving subcutaneously treated adrenalectomized rats the relative potency of 14α-hydroxycortisol was 12% ± 3 (Tables II, IV). When the steroids, as acetates, were administered in the food (test D) the relative potency of the 14α-hydroxy derivative was 19% ± 3 of cortisol (Tables III, IV).

Summary and conclusion. Introduction of the 14α-hydroxy group into cortisol caused a severe reduction in thymolytic activity when assessed by a test employing the adrenalectomized rat, while similar studies in the mouse indicated a thymolytic activity of the order of cortisol. These studies demonstrate that relative activity of 2 corticoids on the thymolytic test can vary as much as 8-fold depending upon the animal and conditions chosen for the bioassay.

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Studies on Distribution of *Escherichia coli* Endotoxin in Mice.* (24525)

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Although physiological changes resulting from their injection have been described, the lethal mechanism of lipopolysaccharide materials derived from gram-negative bacteria is not known. Indeed, until recently, there has been no good qualitative technic for *in vivo* detection of minute qualities of endotoxins. Such a technic has been developed by Braude, *et al.*(1) who have shown that Cr^{51} can be used as a reliable tag. This study was undertaken in the hope that changes in distribution could be correlated to toxicity and that this, in turn, would provide some insight as to mode of action of endotoxins.

Materials and methods. The endotoxin preparation used was prepared from a strain of *E. coli* isolated from a fatal human septicemia. The bacteria were grown in synthetic medium with aeration, harvested by centrifugation and killed and dried with acetone. Endotoxin was removed by extraction with trichloroacetic acid and was further purified by ethyl alcohol-salt (NaCl) fractionation (2). Nitrogen content of endotoxin was 2.3% by weight. All dilutions were made with 0.9% NaCl solution. Tagging of the endotoxin was carried out as follows: Approximately 1 mc of sterile $Cr^{51}Cl_3$ (Abbott Labs., Oak Ridge, Tenn.—specific activity approximately 1 mc/mg) was added to saline solution of 100 mg endotoxin and kept at room temperature. After 24 hours the mixture was dialyzed at room temperature against 5000 ml volumes of 0.067 M phosphate buffer, pH 7.2, which was changed at 24-hour intervals until appearance of Cr^{51} in the dialysate was negligible, usually 72 to 96 hours. Tagging by this procedure did not alter the original toxicity of the endotoxin. All mice were Bagg strain albino, obtained from Walter Reed Army Medical Center colony. The mice were housed in glass jars where food (containing no antibiotics) and water were available. Some mice were injected with trypan blue to alter their susceptibility to endotoxin by intraperitoneal administration of 2.5 mg trypan blue in distilled water daily for 3 successive days. On the fourth day mice were injected with endotoxin. To measure radioactivity, entire organs were removed and each was digested by boiling in 2 ml of concentrated HNO_3. Foaming was prevented by adding small amount of antifoam (Dow Corning Silicone Defoamer—Antifoam A) to each digestion tube. Blood was obtained by cardiac puncture and digested as above. All measurements of radioactivity in blood were based on total blood volume, considered to be 8% of body weight. Radioactivity of each sample was measured in well-type scintillation counter which had an efficiency of approximately 3%. The counting error at the 0.95 level was 23 to 28 cpm for the 2-minute counting intervals.

Results. Comparison of distribution patterns in mice of Cr^{51} tagged endotoxin and free $Cr^{51}Cl_3$ one hour after intravenous administration showed that most Cr^{51} bound to endotoxin was in the liver and remained there for long periods. Free $Cr^{51}Cl_3$ was more generally distributed and appeared early in urine in considerable amounts. Toxicity studies showed that routes of injection greatly influenced lethality of this preparation. The LD_{50} dose was 145 μg intravenously and intracranially, 290 μg intraperitoneally and 580 μg intramuscularly. Distribution studies were carried out to explain these differences.

Distribution of endotoxin following intravenous administration. There was an appreciable rise in amount of endotoxin in the blood when the amount of toxin administered exceeded the lethal level, *i.e.*, 150 μg (Table I). However, it should be noted that this does not necessarily represent overflow endotoxin because it is apparent that amounts in the organ continued to increase with increases in dosage. Using larger groups of mice, showed

* Presented in part before Soc. Am. Bact., Chicago, Ill., Apr. 1958.

TABLE I. Distribution of Cr^{51} Tagged Endotoxin Administered Intravenously in 20-22 g Mice (Sacrificed 3 Hr after Injection).

# of mice	Toxin dose (μg)	Endotoxin in blood (μg)	Endotoxin in liver (μg)
9	1	.1	.69
4	10	.20	7.7
17	100	2.2	62.2
17	150	6.4	79.8
10	175	17.5	86.5
15	200	27.2	103.8
10	225	41.6	131.1

that most injected endotoxin was found in the liver within 5 minutes following injection and the amount detectable remained constant for long periods. An experiment was carried out to determine length of time (as measured by radioactivity) the endotoxin distribution pattern remained unchanged. Three groups of mice were injected intravenously with 1, 5, or 50 μg of endotoxin, sacrificed at intervals, when various organs were checked for radioactivity. Measurable radioactivity in livers and spleens remained constant for 30 days while most of the radioactivity disappeared from blood and lung specimens much earlier. Approximately 60% of injected radioactivity was still present in the liver 60 days after injection of endotoxin.

Distribution of endotoxin following intracranial administration. Using a 27 guage needle, mice weighing 18 to 20 g were injected intracranially with 0.2 ml saline containing varying dosages of endotoxin and sacrificed as described earlier. The LD_{50} dosage of 150 μg was used in most instances. The results showed that distribution pattern of LD_{50} dose by this route was similar to the same LD_{50} dose by intravenous route. Egress from the cranium was rapid. It was not determined whether the 20 to 40% of injected dose remaining in the head was bound by brain tissue. When endotoxin is administered by other routes it is not found in appreciable amounts in the head and in rabbits, at least none is found in the brain. It is of interest to note that dosage and time of death by intracranial route was very similar to dosage and time of death by intravenous route. When the overwhelming dosage of 870 μg/mouse was used, percentages of injected dosages found in blood were again strikingly similar to that found using intravenous administration. It is tempting to postulate a threshold liver or blood concentration above which death from endotoxin occurs and below which survival is the rule. Such a hypothesis, however, did not hold when intramuscular route of administration was used.

Distribution of tagged endotoxin following intramuscular administration. The LD_{50} of this endotoxin prepartaion by intramuscular route was about 580 μg/mouse, which was the dosage used to study distribution by this route of administration. The distribution pattern was quite different by this route than by intravenous or intracranial routes (Table II). From one-third to one-half of the injected toxin remained at site of injection and not more than 4% of the toxin was found in all other areas assayed. Blood levels of endotoxin increased rather than decreased with time but total amount detected in the liver was far less than that following intravenous or intracranial injection. Thus lethality by this route could not be correlated to liver saturation of endotoxin.

Effect of premedication with trypan blue on endotoxin distribution. Administration of trypan blue reportedly blocks the reticuloendothelial system and results in greater susceptibility to endotoxin. The increased susceptibility was confirmed, ranging from increase of 4-fold to 10-fold depending on route of administration of endotoxin. Table III gives a comparison of the distribution pattern in normal mice and mice premedicated with trypan blue when the intravenous route

TABLE II. Distribution of Cr^{51} Tagged *E. coli* Endotoxin* in Normal Mice after Intramuscular† Injection.

# of mice	Time of sacrifice	Endotoxin (μg)			
		Blood	Lungs	Liver	Site of inj.
6	5 min.	.6	.6	3.6	393.2
6	30	1.8	.6	4.8	377.0
4	1 hr	1.8	.6	7.8	372.4
4	2	1.8	.6	5.4	366.0
4	3	1.8	.6	8.4	345.1
6	4	1.8	.6	9.0	335.2
4	5	3.0	.6	13.8	349.2
2	6	3.6	6.6	6.0	315.5
6	24	3.0	.6	14.4	301.0

* 580 μg.
† Inj. vol was 0.2 ml into gluteal area.

TABLE III. Comparison of Endotoxin Distribution in Normal Mice and Mice Premedicated with Trypan Blue.

# of mice	Premedication	Time of sacrifice (hr)	Toxin admin. (μg)	Route of admin.	μg of endotoxin in		
					Blood	Liver	Site of inj.
10	Trypan blue	3	145	I.V.	14.1	66.9	
17	Saline	3	145	"	6.4	79.8	
6	Trypan blue	3	145	I.M.	.1	1.9	
6	*Idem*	3	290	"	.9	2.0	
4	Saline	3	580	"	1.8	8.4	345
5	Trypan blue	24	145	"	3.8	3.9	50
4	*Idem*	24	290	"	5.5	6.1	97
6	Saline	24	580	"	3.0	14.4	301

of administration was used. The amount found in blood was increased in the trypan blue mice, but the amount in other organs assayed was not appreciably altered. When trypan blue mice were injected intramuscularly, distribution 3 hours after injection was similar to that of normal mice. At 24 hours after injection, however, the blood level of trypan blue mice was as great or greater than that of normal mice even when dosage administered to the trypan blue mice was 25% that of normal mice (Table III).

Discussion. A number of technics have been used to detect *in vivo* endotoxins. Braude(3) and his co-workers working with rabbits found that following intravenous administration, endotoxin was present in plasma but not the erythrocytes. It then passed to the buffy coat and liver. Accumulation in the buffy coat was accompanied by a pronounced leucopenia, primarily a fall in lymphocytes and neutrophils. Very little of the endotoxin could be found in spleen or lung and none in the brain. In a continuation of this study Carey *et al.*(4) studied the effect of tolerance on distribution. They found that although normal and tolerant animals both cleared sublethal doses of endotoxin rapidly and at approximately equal rates, a great difference was noted between tolerant and non tolerant animals. Hepatic localization and plasma clearance of massive quantities of endotoxin were much more rapid in tolerant animals. The authors felt that tolerance to lethal doses of endotoxin depends on permanent removal of circulating endotoxin into hepatic and other cells.

Rowley *et al.*(5) found that when 5-60 μg of P^{32} phosphorus labelled endotoxin was injected intravenously into mice, 60 to 75% was eliminated from the circulation in 8 minutes and that at least half of the removed radioactivity appeared in liver and spleen and smaller amounts appeared in lungs, adrenals and kidneys. They noted that endotoxin removed by reticuloendothelial cells appeared to be firmly held by them. Using the fluorescent antibody technic Cremer and Watson(6) studied distribution of endotoxin in rabbits following intravenous injection. In normal animals the toxin was removed from the circulating blood quickly by the reticuloendothelial system. There was a reduction of toxin with time in spleen and lungs, but the liver maintained a rather constant pattern. In cortisone-treated or irradiated animals the amount of endotoxin remained high in spleen and lungs as well as in liver. When premedicated with thorotrast, little or no endotoxin was removed by the reticuloendothelial system and there was an increased death rate.

The above studies suggest that lethality of endotoxin is associated with failure of the reticuloendothelial system to remove circulating endotoxin. Some results in this study tend to support this theory. These are: 1) Partial blockade of the reticuloendothelial system by trypan blue increased susceptibility of mice to endotoxin and resulted in high blood levels of endotoxin. 2) Amounts of circulating endotoxin increased rapidly when lethal amounts of endotoxin were injected via intravenous or intracranial routes. The latter finding, however, does not necessarily represent reticuloendothelial system saturation because the liver continued to take up endotoxin when greater amounts were injected. There is, of course, the possibility that this in-

creased endotoxin is in hepatic cells rather than Kupffer cells.

The relatively constant percentages of radioactivity remaining in liver and spleen suggest a number of possibilities. First, radioactivity in liver represents cleaved Cr^{51}. This seems unlikely because it has been shown that free Cr^{51} does not tend to accumulate in liver or spleen. A second possibility is that the Cr^{51} is attached to some degraded or detoxified endotoxin in these organs. The possibility of a liver "endotoxinase" must be considered. A third possibility is that the cells of the reticuloendothelial system protect the animal by holding the endotoxin and releasing it very slowly over long periods. One finding in this study that is difficult to relate to reticuloendothelial system saturation as a factor in death from endotoxin is the pattern of distribution following intramuscular administration of endotoxin. Amount and distribution of radioactivity, and presumably endotoxin, found in the reticuloendothelial system following intramuscular injection is compatible with life as demonstrated by intravenous injection of sublethal amounts of endotoxin, yet the animals injected intramuscularly, died. One possible explanation is that the tag is cleaved in muscle tissue with the Cr^{51} being excreted or more generally distributed and the Cr^{51}-free endotoxin is present but undetectable in liver. Another possible explanation is that a lethal mechanism of endotoxin is its persistence in the blood. Initially blood levels of endotoxin following intramuscular injection are low, but, in contrast to the pattern seen when other routes of injection were used, levels continued to rise and persist for at least 24 hours. The lethal mechanism of endotoxin may be vascular in this instance. Some evidence for such a time-dose type of theory is that mice rarely die from a barely lethal dose of endotoxin within the first 8 hours after injection and most are dead within 24 hours after injection.

Summary. 1. Intravenous injection of Cr^{51} tagged endotoxin into mice resulted in marked increase of circulating endotoxin as dosages reached the lethal range. 2. Distribution pattern of endotoxin in mice injected intracranially was similar to that of mice injected intravenously. 3. Distribution pattern of endotoxin in mice injected intramuscularly differed markedly from those found in mice injected intravenously or intracranially. 4. Premedication of mice with trypan blue resulted in greater susceptibility to endotoxin and increased amounts of circulating endotoxin in mice injected intramuscularly or intravenously. 5. Using the technique described, lethality of endotoxin for mice could not be related to any one distribution pattern.

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Chromatography of McCaslan Pole Bean Hemagglutinin on DEAE-Cellulose. (24526)

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Phytohemagglutinin of McCaslan Pole Bean (*Phaseolus vulgaris*) has been a material of interest since discovery of its ability to agglutinate human erythrocytes (rbc). Boyd (1) observed that it agglutinates cells of blood groups A, B, and O (all Rh positive). Part of the interest in this material stems from its utility in preparing high purity leukocyte sus-

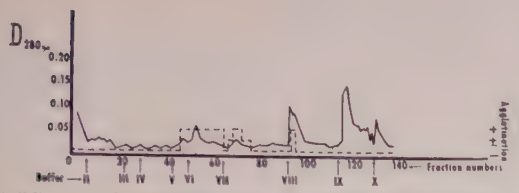

FIG. 1. Chromatogram of phytohemagglutinin. Buffer system (Roman numerals refer to individual buffers and their point of addition to dropping bottle): constant NaCl, 0.0125M; in following sequence M PO_4 and pH are given respectively, I, 0.005, 9.3; II, 0.01, 8.5; III, 0.02, 8.0; IV, 0.04, 7.5; V, 0.08, 7; VI, 0.16, 6; VII, 0.20, 5; VIII, 0.50, 5; IX, 1, 4.5; X, same as IX with NaCl increased to 0.50M. Flow rate, 15 ml/hr. Eluate vol, 2400 ml. D_{280} line ———; agglutination – – – – –.

pensions from peripheral blood samples(2,3). In a study of its purification and properties, Rigas and Osgood(4) found that phytohemagglutinin occurs as a mucoprotein and that hemagglutinating ability remains with protein after removal of polysaccharide. This report is concerned with further characterization of this substance by ion-exchange chromatography on the DEAE-cellulose of Sober and Peterson(5,6) under conditions with which we have studied a number of protein systems. Hemagglutinin distribution was determined by reacting column fractions with a panel of human rbc.

Methods. DEAE-cellulose (Brown Co., type 20, 100-300 mesh) with exchange capacity of 0.4 meq/g was prepared by serially washing 3 times with 0.1N NaOH, water, and 0.1N H_3PO_4, followed by a final wash with 0.1N NaOH. About 10 g in hydroxide form was brought to pH 9.3 with 0.005M H_3PO_4 containing 0.0125M NaCl. Equilibration was completed by washing twice by decantation with 21 buffer I (Fig. 1). Exchanger, poured into the column (10 mm i.d.) as a slurry, was packed under N_2 pressure (15-20 p.s.i.) to a height of 15 cm. Gradient elution was conducted by adding each buffer from an inverted bottle with a glass tube dipping beneath the liquid surface of a 500 ml Wolff bottle, which served as mixing chamber. The latter, fitted tightly to the column, was agitated with magnetic stirrer. A crude preparation of phytohemagglutinin was made by the method of Boyd(1) and was thoroughly dialyzed *vs.* buffer I before being added to the column. Chromatography was conducted on 3 ml of this material characterized by a titer of 1/512 with fresh rbc of varying blood group and Rh types. Chromatogram evaluation (Fig. 1) was by optical density measurement on individual fractions at 280 mμ (Beckman DU) and by agglutination tests with a panel of rbc from 5 donors, selected for a variety of blood group combinations. Rbc were typed for the following factors: A, B, O, C, D, E, c, e, C^w, D^u, E^u, M, N, P, Fy^a, Jk^a. These tests were performed by adding one drop of 2% rbc suspension (in saline) to one drop of chromatographic fraction in 10 x 75 mm test tube; tests were incubated at 25°C, 1 hr, and read microscopically. After overnight refrigeration, they were re-read microscopically. Fractions that gave agglutinates at first reading were scored +, those that were positive only after prolonged standing were scored ±.

Results. Fig. 1 reveals fractionation of the protein charge, as evidenced by several optical density peaks. Protein recovery by this chromatographic technic has been consistently 85-90%. Agglutinin was found in fractions 46-63, 66-75, and 93-94; several fractions on either side of 70 and 71 gave positive reactions only after overnight refrigeration. Each of the 5 cell types used in testing reacted equally well in each instance that positive reaction was observed. Titers were obtained for the 3 different groups of chromatographic fractions in which agglutinin was demonstrable; for fractions 52, 70 and 93, titers were 1/4, 1/1, 1/2, respectively. Titrations were identical with each of the 5 cells used. Because of dilution of activity by the large eluate volume, no fraction had a titer as high as that of the charge despite the significant protein concentration in fractions with no hemagglutinating activity.

Discussion. The several absorbance peaks in the chromatogram give evidence of significant protein fractionation. The elution system (previously described for serum protein chromatography(7)) differs from that utilized by Sober *et al.*(6) and Fahey *et al.*(8), who have made extensive studies with DEAE-cellulose, in that higher initial pH and ionic

strength are used. We found that these eluting conditions prevent break-through of serum gamma globulins. Like human anti-A, anti-B, and anti-D(7), phytohemagglutinin is heterogeneous under these chromatographic conditions. In fractions exhibiting activity, agglutination was observed with each test rbc. When titrated *vs.* the rbc panel, crude bean extract and fractions from the 3 activity peaks gave the same titers with each rbc used. Thus, despite fractionation of hemagglutinating activity, it appears that panagglutinin character is preserved qualitatively and quantitatively in each segment of the chromatogram.

Summary. A crude preparation of McCaslan Pole Bean phytohemagglutinin has been fractionated chromatographically on DEAE-cellulose. Hemagglutinating activity, followed by testing each fraction with an rbc panel, was found in 3 groups of fractions. In each of these groups the activity was panagglutinating and gave the same titers with each rbc used in testing.

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Effect of Growth Hormone Upon Thyroid Secretion Rate in the Rat.* (24527)

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It has been shown that thyroid hormone output increases during growth(1) and lactation(2) in the rat. Growth hormone (GH), in addition to its well known ability to cause an increase in body weight(3), has been demonstrated to cause an increase in milk secretion in this species(4). It seemed of interest, therefore, to determine if these effects of GH were associated with an alteration in thyroid activity.

Materials and methods. Adult virgin female rats of Sprague-Dawley-Rolfsmeyer strain weighing 240-300 g were kept under conditions of uniform temperature (78 ± 1° F) in room artificially illuminated during normal daylight hours for 5-6 weeks prior to experiments. Some were bred with male rats of same strain during this time. Each of 29 litters obtained was reduced to 6 young shortly after birth and, on 4th day postpartum, each mother was injected i.p. with 2 μc carrier-free I^{131}. Thirty-five non-lactating rats were similarly injected. Forty-eight hours were allowed for fixation of I^{131} by the thyroid and for urinary elimination of excess isotope. External thyroid counts were taken at this time and at either 24 or 48 hour intervals thereafter by first anaesthetizing each animal with ether, then placing it on a lead plate with its thyroid region over a scintillation probe. Measurements of thyroidal radioactivity were made with scintillation counter, Nuclear-Chicago (N.C. Model DS 5) connected to pulse height analyzer (N.C. Model 1810) which, in turn, was connected to a rate meter (N.C. Model 1620A). Conventional corrections were made for radioactive decay and background. *Measurement of thyroid secretion rate (TSR).* Twenty-four lactating and 15 non-lactating rats were injected subc.

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† Postdoctoral Fellow of N.I.H. This investigation was supported in part by grants from P.H.S.

with GH‡ at 1 mg/day commencing 3 days after injection of I^{131} (7th day postpartum for lactating animals). The non-lactating rats were injected subc. with L-thyroxine at .5 μg/100g/day for 2 consecutive days starting 4 days after I^{131}. The dose was increased at .5 μg/100g increments, each level for 2 consecutive days with thyroid count taken the day of each increase. Each lactating rat was similarly treated with the exception that initial dose of L-thyroxine was either .5 or 1.0 μg/100 g and the dose increased at 1 μg/100 g increments. TSR was determined by plotting percentage of previous count with thyroxine dose. The dose which prevented further thyroidal I^{131} output in each rat (95-100% of previous count) was estimated as its TSR. In lactating rats, extrapolation to 100% of previous count was necessary when TSR was not obtained by day 16 postpartum. Average TSR of each group was compared with control values previously obtained with the same strain of rats under identical conditions(2). *Effect of GH upon rate of release of thyroidal I^{131}.* Twenty non-lactating and 5 lactating rats were injected daily 8 days commencing 48 hours after I^{131} injection with levels of L-thyroxine (3 and 3.5 μg/100 g for non-lactating and lactating rats, respectively) which exceeded by .5 μg/100 g in each case the highest TSR previously observed(2). External thyroid counts were made every 24 hours during this period, the last count obtained 24 hours after last injection. The animals were treated subseqeuntly as follows: 1) Five non-lactating rats received only L-thyroxine; 2) Five non-lactating and 5 lactating rats were injected with 1 mg/day GH for 4 days starting the 4th day of thyroxine treatment; 3) Since each mg of GH contained approximately .008 USP thyrotropin (TSH), 5 non-lactating were injected with this level and 5 with .024 USP TSH§/day for 4 days starting 4th day of thyroxine treatment. Rates of thyroidal I^{131} release during each 4 day control and experimental period were based upon the calculated slope of each regression line. Significance of difference between control and experimental slopes in each group was estimated by the method of analysis of variance.

TABLE I. Effect of GH upon Thyroid Secretion Rate in Rats.

Treatment	No. of rats	Avg Body wt (g)	Avg TSR (μg/100 g L-thyroxine)
Non-lactating control*	20	262.0	1.3 ± .11
Idem + 1 mg GH/day	15	245.4	1.2 ± .13
Lactating control*	16	285.3	2.2 ± .22[1]
Idem + 1 mg GH/day	24	285.3	2.9 ± .12[2]

* Data from Grosvenor and Turner(2).

Student's "t"	Probability
1–2	.01

Results. Body weight of non-lactating and lactating rats injected with 1 mg GH/day increased 2 g/day in comparison with no gain for control non-lactating and .5 g/day for lactating rats during the same interval. Average TSR of non-lactating rats injected with GH was not significantly altered from the control value of 1.3 μg/100 g L-thyroxine (Table I). During days 8-16 postpartum, the average TSR of 2.9 μg/100 g for lactating rats receiving GH was 32% greater than the value of 2.2 μg/100 g obtained for untreated lactating rats. The difference was significant ($P = .01$). Thyroidal I^{131} output was almost totally prevented in non-lactating and lactating rats by daily administration of L-thyroxine which exceeded their TSR (Fig. 1). No change in rate of thyroidal I^{131} output of non-lactating rats resulted from subsequent administration of 1 mg GH or from TSH in amounts equal to or 3x greater than that present in 1 mg GH. A significant increase ($P = .01$) in rate of thyroidal I^{131} output occurred, however, in lactating rats injected with 1 mg GH.

Discussion. The influence of GH upon thyroid activity has received little attention. It has been shown GH significantly reduced thyroidal uptake of I^{131} and tended to decrease serum I^{131} in adult rats though an increase in body weight occurred(5). In our study, GH evoked gains in body weight on the average

‡ The growth hormone (NIH-BGH-1) is a highly purified bovine preparation distributed by the Nat. Inst. Health.

§ Reference Standard containing .074 USP TSH/mg.

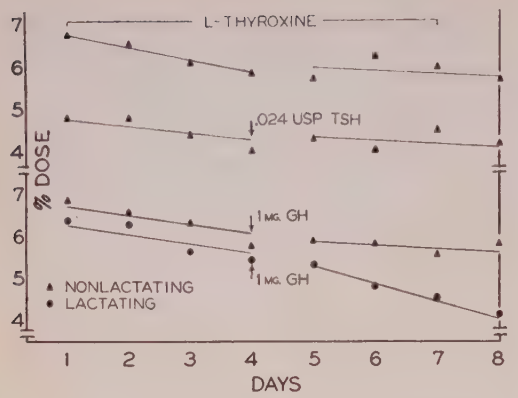

FIG. 1. Effect of GH and TSH upon rate of release of thyroidal I^{131} in rats whose normal thyroid hormone output has been almost totally prevented by daily administration of L-thyroxine in doses (3.0 and 3.5 μg/100 g for non-lactating and lactating rats, respectively) which exceed daily thyroid secretion rate. Each slope represents avg of 5 rats.

of 2 g/day, but failed to alter TSR or increase rate of thyroidal I^{131} release in mature female rats. It would appear that ability of GH to increase body weight does not involve an alteration in thyroid activity though it is well known that thyroid hormones play an important role in the growth process through their control of cellular metabolism.

Increased milk yields have been shown to result from administration of GH to lactating rats(4), cows(6,7), goats(8) and sheep (9) presumably because of favorable influences exerted upon protein, carbohydrate and fat metabolism. In the present investigation significant increases in TSR and rate of thyroidal I^{131} release occurred in lactating rats injected with GH. This suggests that a part of the galactopoietic action of GH in lactating rats may be due to increased levels of thyroid hormones, as a mild hyperthyroidism is generally thought to be desirable for optimal milk secretion(10). This would assign to GH ability to cause an increased secretion and/or release of TSH or thyroxine. There was no indication, however, that these effects were caused by the slight contamination of GH with TSH since the latter in amounts 3x that present in 1 mg of the GH preparation failed to alter release rate of thyroidal I^{131} or affect TSR of non-lactating rats. The data in the present study may be interpreted also as suggesting that elevated thyroid activity is an effect rather than a cause of increased milk secretion due to GH. A concomitant increase in metabolic demands by the mammary gland, imposed by GH, may so increase thyroxine requirements for control of cellular metabolism that the pituitary-thyroid servomechanism may become adjusted to operate at a higher level of activity.

Summary. The effect of 1 mg/day GH upon thyroid activity has been studied in non-lactating and lactating rats. GH did not alter thyroid secretion (TSR) or affect rate of release of thyroidal I^{131} in non-lactating rats though body weight was increased 2 g/day. During days 8-16 of lactation, the average TSR of 2.9 μg/100 g L-thyroxine for lactating rats receiving GH was significantly greater than the 2.2 μg/100 g obtained for untreated lactating rats. Rate of release of thyroidal I^{131} also was significantly increased suggesting GH in some manner effects an increase in output of TSH or thyroid hormones in the lactating rat.

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Influence of Hypothalamus on Pituitary-Thyroid Axis in the Rat.*† (24528)

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We have presented data indicating that lesions in the anterior hypothalamus of the rat produced effects which could be ascribed to reduction in rate of synthesis by the pituitary gland of a single thyrotropic hormone (TSH)(1). Our data did not support Greer's hypothesis(2) which called upon 2 separate thyrotropins, a "metabolic" and a "growth" factor, to explain normal iodide trapping together with reduced hypertrophy after goitrogen treatment in appropriately lesioned rats. Our data showed a reduction in a number of thyroid function criteria. However, we were able to bring only indirect evidence that TSH secretion was decreased by anterior hypothalamic lesions. More direct evidence has been reported by D'Angelo and Traum(3) but the nature of their bioassay makes it impossible to argue convincingly against the dual hormone hypothesis on the basis of their experiments. The current work presents direct evidence that in the lesioned rat there is reduced secretion of "metabolic" TSH. We have further attempted to elucidate the nature of hypothalamic intervention in TSH secretion via the pituitary-thyroid "feedback" mechanism, and we have expanded on previous observations on the effect of hypothalamic lesions on distribution of iodinated amino acids within the thyroid gland.

Methods. Rats used were adult males of Holtzman strain weighing about 250 g at time of lesioning. Large bilaterally symmetrical lesions were produced in the anterior hypothalamic area controlling thyroid function by the procedure previously described(1). Hyperphagia occurred in more than half of the survivors. Rats were used at least a month after lesioning. Propylthiouracil was added to the diet at a level of 0.15% when indicated. Uptake of radioiodine into the thyroid and discharge of radioactivity from this gland were measured as described previously. The thyrotropin biossay employed was developed in this laboratory(4) and is a modification of Gilliland and Strudwick's method measuring discharge of labelled hormone in the chick. Thyroid hormone secretion was measured by estimating graphically the minimum dose of thyroxine given intramuscularly which would completely inhibit discharge of radioactivity from the rat thyroid. Paper chromatography was carried out by the ascending technic at 23° using collidine-water in an atmosphere of ammonia, tert-amylalcohol-acetone-water (20:5:3) and N-butanol saturated with 2 N formic acid. Chromatograms were cut into 1 cm segments at right angles to the direction of migration, and radioactivity determined by means of a well-type scintillation counter.

Results. Table I summarizes results of assaying the pooled sera of 5 "goiter block" and 8 control rats which had been given PTU for 16 days. The serum was administered to chicks in two-1 cc doses. Sera of a group of 5 rats which had been lesioned, but in which the lesions proved to be "ineffective" as to thyroid function by criteria established previously(1), proved to give effects intermediate between "goiter block" and control animals. Since the bioassay employed is based upon a clearly "metabolic" effect, the discharge of labelled hormonal iodine from the thyroid gland, these results show that the level of circulating "metabolic" TSH is lower

TABLE I. Serum TSH Levels in Rats on PTU Diet.

Groups receiving:	No. chicks	% of thyroidal I^{131} discharged
Saline	9	33.9 ± 3.2*
5 mU TSH (USP standard)	10	57.3 ± 4.0
20 *Idem*	10	81.9 ± 3.0
"Goiter block" serum	11	33.3 ± 1.8
"Ineffective" lesion serum	9	45.3 ± 1.1
Unlesioned control "	11	52.7 ± 4.0

* Stand. error of mean.

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in goiter block animals than in controls or rats lesioned in areas other than the thyroid controlling one. This difference was significant at the .001 level of confidence.

Unfortunately we have been unable to duplicate these experiments in rats which had not been treated with goitrogens because the levels of TSH in normal serum still appear too low to be determined by available bioassay procedures. Taken in conjunction with our previous findings, the data would seem to obviate the need for assuming the existence of 2 thyrotropins. A single hormone produced under partial hypothalamic control would suffice to explain all data presently available except the work on hypophysectomized mice bearing intraocular pituitary grafts reported by Scow and Greer(5). It may well be that sensitivity to TSH of different aspects of thyroid function varies among different species, and for this reason we are currently reexamining the influence of autografted pituitaries on rat thyroid function in collaboration with Dr. K. M. Knigge.

Von Euler(6) first showed that in the rabbit the site of feedback control of TSH release was in the pituitary rather than the hypothalamus. Brown-Grant(7) postulated that since the majority of hypophyseal blood supply was routed through the hypothalamus, the latter might act as a filter, keeping thyroxine from reaching the pituitary and thus maintaining TSH secretion at a high level. It was therefore of some interest to determine whether appropriate hypothalamic lesions would alter the amount of thyroxine needed to inhibit thyroidal I^{131} discharge completely. In a preliminary experiment 4 out of 15 control rats responded to .2 γ thyroxine a day and 11 to 4 γ. Among lesioned rats 4 out of 14 responded to 2 γ and 10 to 4 γ. In a second experiment using the same animals a month later the stepwise increments were made smaller and a more quantitative estimate was obtained for the minimum effective dose. The effective dose was 2.8 ± 0.2 (standard error) γ for controls and 2.6 ± 0.3 γ for lesioned animals. There was no difference between the 2 groups and we are in agreement with D'Angelo and Traum(8) who also believe that thyroxine inhibits TSH secretion at the pituitary without hypothalamic mediation. The hypothalamus thus would appear to act primarily upon synthesis of TSH rather than its release as claimed by Shibusawa(9), since we previously showed below-normal TSH stores in pituitaries of lesioned animals.

In our previous studies of iodine content of thyroids of normal and lesioned rats, we were unable to show significant differences in I^{127} content between the 2 groups. This was interpreted as arguing against the action of the thyroid-controlling mechanism independent of the pituitary reported by VanderLaan and Caplan(10). However, Blanquet and Tobias(11) have found lesions produced in rat hypothalami by means of a cyclotron beam to cause a striking decrease in thyroid hormone production without concomitant decrease in other organic iodine binding. We have therefore examined pancreatin digests of I^{131}-labelled thyroids from lesioned and control rats by ascending paper chromatography using 3 different solvent systems: collidine-ammonia, tert-amyl alcohol-acetone-water, and n-butanol-formic acid-water. The first 2 systems were used on groups of 9 lesioned and 9 control rats which were killed 24 hours after tracer administration. The third system was used on 19 glands from animals taken 72 hours after tracer administration. In no case could major differences in the distribution of I^{131} between iodide, iodotyrosines and hormonal iodine be observed. This work is being repeated in collaboration with Drs. P. Blanquet and D. Yudilevich using both paper chromatography and their ion exchange method in parallel on the same thyroid digests.

Summary. In rats with anterior hypothalamic lesions the maximal circulating thyrotropin levels after goitrogen feeding are lower than in control animals as measured by bioassay based upon hormonal iodine discharge from chick thyroids. The discharge of labelled hormone from the thyroid of lesioned and control rats is inhibited by equal doses of exogenous thyroxine. This indicates that the thyroid-pituitary feedback system controlling TSH release is independent of the hypothalamus. Finally, in electrolytically lesioned rats with destruction of the thyroid-controll-

ing hypothalamic area there do not appear to be major changes in the relative amounts of iodinated tyrosines and thyronines in the thyroid as have been shown to occur in cyclotron-lesioned rats by Blanquet and Tobias.

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Aromatic Amines II. Demonstration of 2-amino-1-naphthyl Glucuronides as Metabolite of 2-naphthylamine in Dog Urine.* (24529)

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Occupational exposure to 2-naphthylamine is recognized as producing bladder cancer in man(1). Attempts to produce bladder cancer in experimental animals with this compound have shown that the dog develops bladder cancer(2,3) with pathology similar to humans(2), while other species such as rabbits, rats, and mice do not(3). Rabbits and rats develop benign tumors but mice show no bladder damage(3). When 2-naphthylamine and some of its metabolites were implanted in the bladder of the resistant mouse, it was found that certain metabolites led to bladder cancer. Of the 7 metabolites tested, 2-amino-1-naphthol hydrochloride(3,4), and 2-amino-1-naphthyl glucuronide(5) were active in producing bladder carcinoma. This observation supports the hypothesis(2) that species specificity is linked to metabolites produced rather than to species difference in sensitivity of bladder tissue. This hypothesis requires that after exposure of 2-naphthylamine the carcinogenic metabolites must be present in urine of susceptible species in significantly higher concentration. The main metabolite of 2-naphthylamine in the dog is 2-amino-1-naphthyl sulfate(6). Since this appears to be inactive in the mouse bladder(4), a search was undertaken for other metabolites in dog's urine. Recent work in this laboratory demonstrated the presence of a previously unreported metabolite in dog urine, bis(2-amino-l-naphthyl) phosphate(7) however, its carcinogenicity has not yet been determined. In the present communication evidence is presented that the dog excretes 3.5-13% of ingested 2-naphthylamine as 2-amino-1-naphthyl glucuronide.

Materials. 1. *2-amino-l-naphthol hydrochloride.* Chromogene Black ETOO (General Aniline and Film Co.) was reduced with sodium hydrosulfite as described by Grandmougin(8). The product was extracted with ether containing a small amount of hydroquinone. The dried ethereal solution was treated with gaseous hydrogen chloride to give the desired product. The infrared spectrum (KBr wafer) was identical with that of a 2-amino-l-naphthol hydrochloride sample generously supplied by the National Aniline Di-

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vision. 2. *2-amino-l-naphthyl glucuronide* prepared biosynthetically in the rabbit as described by Boyland and Manson(9). 3. *1,2-naphthoquinone-4-sulfonate (NQS)* recrystallized from commercial material(10).

Methods. 1. *Administration of 2-naphthylamine.* One g of 2-naphthylamine in 000 gelatin capsules was fed daily to 4 female dogs in the weight range 19-22 kg. Urine was collected daily under toluene. 2. *Determination of aromatic amines* by reaction with NQS (14). Urine samples were brought to pH 2 to 4 and a fivefold excess of 0.02 M NQS was added. The solution was extracted with 3 ml of benzyl alcohol. The benzyl alcohol was washed 3 times with equal volumes of 0.01 M hydrochloric acid. Aliquots were diluted with ethanol and absorption determined at 470 mμ in Beckman spectrophotometer. 3. *Determination of 2-amino-l-naphthol* by method developed for identification and measurement, which depends on its oxidation to ortho naphthoquinone and subsequent coupling with aniline. Further details of this method and its application to other ortho amino phenols will be presented elsewhere. One ml samples containing 0.2-2 μM of 2-amino-l-naphthol in 0.1 N $Na_2S_2O_4$ were extracted with 3 ml of benzyl alcohol containing 0.1 M aniline. The benzyl alcohol layer was treated with 1 ml 1 M $FeCl_3$ in 0.1 N hydrochloric acid and the solution shaken vigorously 2 minutes. The red 4-anil-1,2-quinone was determined in Beckman spectrophotometer at 480 mμ after dilution with ethanol. 4. *2-amino-l-naphthyl glucuronide.* Samples in 0.05 M $Na_2S_2O_4$ were adjusted to pH 5.2 and divided into 2 parts. Each received 0.1 volume of 0.1 M acetate buffer (pH 5.2) and 1000 units/ml of β-glucuronidase (ketodase, Warner Chilcott and Co., N. Y.). To one sample, was added a solution of saccharic acid which has been boiled 30 minutes at pH 3.5(11). Final concentration was 0.002 M with respect to saccharate. Each sample was incubated at 37° for 4 hours with aliquots taken at desired intervals for assay of 2-amino-l-naphthol.

Results. When 1 g of 2-naphthylamine was fed daily to dogs, primary aromatic amine metabolites were typically excreted as shown in Table I. 2-amino-l-naphthyl glucuronide comprised a significant portion of the amine fraction. Its presence was demonstrated by combination of paper chromatography and β-glucuronidase action.

TABLE I. Excretion of Total Amine and of 2-amino-l-naphthyl Glucuronide by 20 kg Dog Ingesting 1 g 2-naphthylamine/Day.

Date of collection*	Total amine, mg	2-amino-l-naphthyl glucuronide	
		mg	%/dose
5/ 7/58	985	41.8	4.2
8	535	84.2	8.4
9	545	50.0	5.0
22	812	35.0	3.5
29	1000	133.0	13.3

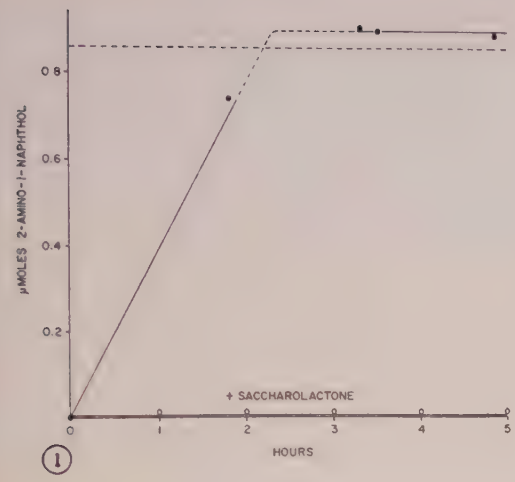
* The dog received 1 g of 2-naphthylamine/day commencing 1/22/58. 2-amino-l-naphthyl glucuronide was determined only on these days.

The glucuronide was extracted from urine at pH 3.5 with n-butanol. After extraction with 0.1 N sodium bicarbonate, the aqueous phase was applied directly on Whatman No. 3 paper. The solvent systems were n-butanol/n-propanol/water (2:1:1)(12) or n-butanol saturated with 1 N ammonium hydroxide.

In each system a prominent fluorescent spot was obtained which gave a positive NQS test and which corresponded in R_F to the authentic 2-amino-l-naphthyl glucuronide. R_F values in the 2 systems were 0.20 and 0.25 respectively. The fluorescent zone was cut out and extracted continuously with methanol. The ratio of amine (NQS method) to glucuronide (carbazole method)(13) in the extract was unity. Furthermore, 1 mole of 2-amino-l-naphthol/mole of amine was released by β-glucuronidase in 2 hours (Fig. 1). This release was completely prevented by the 0.002 M saccharate solution described above which is a specific β-glucuronidase inhibitor(11).

The presence of l-glucuronide was chromatographically confirmed in all 4 dogs. Isolation of the compound and quantitative estimates were made with 2 dogs.

Urine samples were also treated directly with β-glucuronidase. Similar ratios of release of 2-amino-l-naphthol were observed. The results of a typical experiment are presented in Fig. 2. β-glucuronidase inhibition by saccharate was again observed.



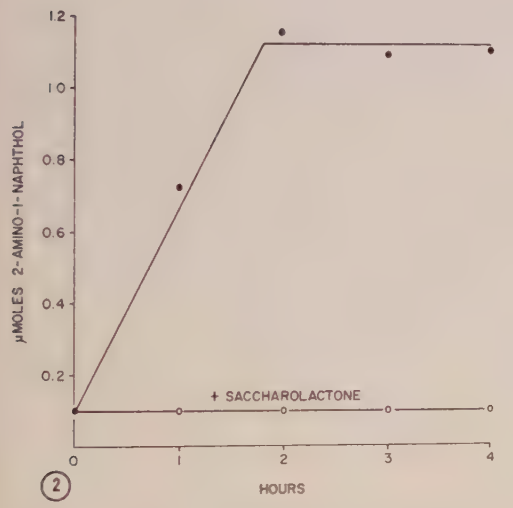


FIG. 1. Action of β-glucuronidase, in presence and absence of saccharolactone, on paper chromatographic eluate of zone corresponding to 2-amino-1-naphthyl glucuronide. From urine of dog fed 2-naphthylamine. – – – – –, total aromatic amines as measured with sodium naphthoquinone sulfonate.

FIG. 2. Action of β-glucuronidase, in presence and absence of saccharolactone, on urine of dog dosed with 2-naphthylamine.

The presence of free 2-amino-1-naphthol was frequently demonstrated in urine. Amounts found were about 2% of administered 2-naphthylamine.

Discussion. It has been previously demonstrated that 2-amino-l-naphthyl sulfate(6) and bis(2-amino-l-naphthyl) phosphate(7) are metabolites of 2-naphthylamine in the dog. Hueper(2) reported frequent appearance of a purple pigment in dog's urine. This pigment, which we have also observed, was undoubtedly an oxidation product of 2-amino-l-naphthol. Additional information is that 2-amino-l-naphthyl glucuronide is also a metabolite.

The origin of the small amount of 2-amino-l-naphthol found in dog urine is of particular interest. We are presently investigating whether it is excreted as such or whether it arises from hydrolysis of unstable conjugates. Concentration of 2-amino-l-naphthol was increased in alkaline urines. The glucuronide and sulfate esters are unlikely precursors, since they are stable to alkaline hydrolysis and are not hydrolyzed by urinary enzymes in alkaline media. There is as yet no definite information available on presence of this metabolite in urine of species resistant to cancer induction.

The demonstration of the presence of these 2 metabolites of 2-naphthylamine in dog urine which are carcinogenic by mouse bladder implantation test, is consistent with the hypothesis that metabolic products of the dog are responsible for its sensitivity to this disease.

However, inconsistent and requiring further scrutiny is the finding of 2-amino-l-naphthyl glucuronide in undefined amounts in the resistant rat and rabbit(9).

Summary. 2-amino-l-naphthyl glucuronide was demonstrated to be a urinary metabolite in dogs fed 2-naphthylamine by identification of the material with the aid of chromatography and enzymatic technics. 3.5-13.3% of ingested dose is excreted in this form when dogs are fed 1 g/day. The possible relation of this glucuronide to bladder cancer is discussed.

The contribution of Miss Joan Royce and Mr. Paul Miles to this work is gratefully acknowledged.

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Effects of Prenatal X-Irradiation on Discrimination Learning in the Rat. (24530)

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It is well established that x-irradiation during prenatal development may result in death, retarded growth and morphological abnormalities, the exact type and magnitude of the effect depending on embryonic stage irradiated and dose used [see review by Russell (1)]. Prenatal x-irradiation also affects maze learning performance of rats. Levinson(2) irradiated rats on 11th to 19th days of pregnancy with single sublethal exposures of x-irradiation and tested offspring when 50 days old on Lashley Type III maze. Learning measured in terms of number of trials to reach a criterion, number of errors and time spent in maze, was impaired with deficits directly related to radiation dose. Radiation on 13th day produced greatest changes. This agrees roughly with Hick's timetable for cortical damage (3). Similar findings were also reported by Tait *et al.*(4) and more recently by Furchtgott *et al.*(5). Data are here presented on effects of x-irradiation during various stages of prenatal development (10th, 14th and 18th day of pregnancy) on behavioral performance on another type of task, *i.e.,* learning a brightness discrimination.

Procedure. Fifty female rats of Long-Evans strain, raised from weaning on natural food stock ration,‡ were selected at 3 to 4 months of age and average body weight of 188 g. Animals were divided at random into 5 equal groups of 10 rats each and mated to males of proven fertility. Pregnancy was dated by finding sperm in vaginal smear.§ Animals in Group I served as non-irradiated controls: those in Groups II, III and IV received a single exposure of 150 r x-irradiation on 10th, 14th or 18th day of pregnancy respectively. Group V received a single exposure of 300 r on 18th day of pregnancy.‖ Radiation factors were as follows: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic fil-

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‡ Rockland Rat Diet, Arcady Farms Milling Co., Chicago, Ill.

§ Start of pregnancy was dated from time that sperm was actually observed in vaginal smear. Inasmuch as mating may have occurred at any time from 5 p.m. of the previous day to 8 a.m. of the day sperm were found, fertilization may have occurred as much as 15 hours before the time that sperm were observed. First day of pregnancy covered the first 24 hours following finding of sperm. It is estimated that actual time of x-irradiation in our experiment was approximately 9½, 13½ and 17½ days post fertilization.

‖ Two additional groups of 10 rats each receiving a single exposure of 300 r x-irradiation on 10th and 14th day of pregnancy were also included. No litters were born to any rats in 10 day 300 r group. In 14 day 300 r group, 7 litters were born but all young died within 5 days of birth.

ter;¶ HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and exposure dose rate, 17.92 r/minute (measured in air, at top of box). Animals to be irradiated were placed in wooden box divided into 14 compartments 7 cm wide, 16 cm long and 10 cm deep. Partitions and top were made of ⅛″ cellulose acetate sheeting; and top, one side and bottom of each compartment were perforated with holes for ventilation. The container was rotated slowly on electrically-driven turntable to insure equivalent irradiation. Throughout pregnancy and lactation, rats were continued on same ration they had received since weaning. Food and water were provided *ad lib*. In Groups I to V, proportion of females casting litters was 90%, 100%, 100%, 90% and 80%; average litter size was 7.8, 7.6, 9.3, 7.7 and 7.3; average birth weight of young, 6.1 g, 5.4 g, 5.5 g, 5.6 g and 5.2 g; and mortality of young during lactation, 7.0, 39.5, 9.6, 9.8 and 13.0%, respectively. Thus, the group varied significantly only in the last parameter. At weaning the young appeared normal except for offspring of group II. The latter in accordance with previous findings(1) exhibited a number of abnormalities including microphthalmia and anophthalmia (present in approximately 40% of group), hydrocephalus (grossly discernible in approximately 20%), paralysis of hind limbs (noted in approximately 10%) and shortening and kinking of tail (approximately 10%). At weaning 10 female rats of comparable size were selected from offspring of Groups I to V respectively. These consisted of at least one animal from each litter weaned. The offspring selected from Group II were carefully screened to eliminate those with grossly detectable defects. Group designations A, B, C, D, E have been applied to young from Groups I to V respectively. These rats were continued on previous diet until approximately 180 days of age, then subjected to a brightness discrimination problem. Body weight of rats in the various groups at this time was as follows: 261 g; 246 g; 209 g; 224 g; and 226 g respectively.

Training procedure. The task was to solve a brightness discrimination problem in which a dim light in the box indicated that the animal could drink freely; a bright light indicated that the drinking tube was electrified and animals attempting to drink would receive a shock. Preliminary to actual training all animals were placed on 23-hour water deprivation schedule. For 2 days they were allowed to drink in the apparatus for one hour *ad lib*, when the signal light would remain dim. During the learning phase the signal light would alternate between periods of low (dim light) and high (bright light) intensity. Dim light indicated a non-shock period while bright light indicated a shock period. Thus, the animal received shock only if it drank while the bright light was on. The bright light preceded the shock phase by 2 seconds. The time sequence control was set for a 10-minute cycle of alternating periods of shock and non-shock for the following minutes: 2, 1, 1, 3, 2, 1. This cycle was repeated 6 times during the training hour. Each day's training began at a different point on the cycle to insure against the animal learning the sequence. Animals remained in box for one hour each day, until they reached and maintained at least 80% correct responses for 2 consecutive days. Any drinking during a non-shock period was considered a correct response, while drinking or attempt to drink during a shock period was classified as incorrect response. Animals were tested daily for 12 consecutive days.

Apparatus. A training compartment 9″ wide, 8″ high and 14″ in length was constructed of ¾″ plywood. The floor was covered with a single wire grid resting ½″ above floor of box. Entrance to training compartment was a hinged door with a wire observation window covered during training trials to make the box light tight. A 1″ diameter signal light with a white frosted filter was located in middle of wall opposite the entrance, 3″ below top of compartment. On the same wall and 3″ directly below signal light, a ½″ copper drinking tube entered the com-

¶ A non-uniform filter which produces a flat isodose surface of x-ray intensity constructed by method of Greenfield and Hand(6). We are indebted to Dr. M. Greenfield and Katherine Hand of Atomic Energy Project, Univ. of California at Los Angeles, for construction of parabolic filter. Center of filter had thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

partment. A 250 cc water bottle was suspended outside the compartment behind back wall. Brightness of signal light was controlled by a rheostat. The copper drinking tube was electrified in such a fashion that when charged the animal received a shock when he completed the circuit from the grid on the floor to the copper drinking tube. The current was controlled by an electronic constant current device. A current of 0.4 milliamperes was used. Recording of drinking was accomplished by a 6-volt circuit which registered the breaking of mechanical contacts when drinking tube was touched. Time sequences for shock and non-shock periods were controlled by a synchronous motor driven cam and microswitches.

Results. The performance of rats in various groups in terms of correct responses is summarized in Fig. 1. The offspring of non-irradiated rats (Group A) reached criterion (minimum of 80% correct responses for 2 consecutive days) on day 9. The offspring of rats administered a single dose of 150 r on 10th or 18th day of pregnancy (Groups B and D) reached criterion a day later (i.e., 10th day). Offspring of rats administered 150 r on 14th day of pregnancy (Group C) or 300 r on 18th day of pregnancy (Group E) had not yet attained criterion after 12 days of testing. On 13th day the latter 2 groups were exposed to absolute discrimination problem (*i.e.*, presence *vs.* absence of light) and the tests continued for additional 4 days. Criterion was still not attained by rats in these groups after the additional 4 days under these conditions.

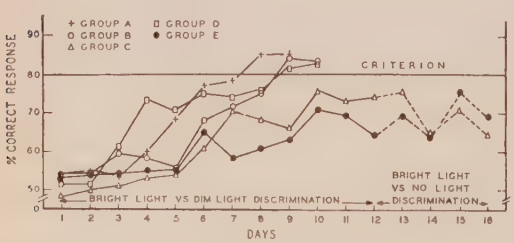

FIG. 1. Discrimination learning performance of rats in Groups A-E. Group A, offspring of non-irradiated rats. Groups B, C and D, offspring of rats which received a single exposure of 150 r x-irradiation on 10th, 14th or 18th day of pregnancy respectively. Group E, offspring of rats administered single dose of 300 r x-irradiation on 18th day of pregnancy.

TABLE I. Means and Stand. Dev. of Errors for Experimental and Control Groups on Day 10 (N = 10 for All Groups).

Groups	Mean	Stand. dev.
A	2.5	1.02
B	3.1	1.37
C	6.1	1.45
D	3.5	1.50
E	6.6	1.49

TABLE II. Analysis of Variance of Error Scores for All Groups.

Sources of variation	df	Variance	F
Between groups	4	34.58	16.31*
Within "	45	2.12	
Total	49		

* Significant at .001 level of confidence.

An error was defined as inappropriate response to the stimulus, *i.e.*, attempting to drink during the bright light (shock) phase of a trial. A trial consisted of a dim light-bright light sequence. There were 18 such sequences a day. A summary of mean number of errors for various groups during the regular 1 hour training period on day 10 (when offspring of non-irradiated rats met criterion) is presented in Table I. A one way analysis of variance of the number of errors on day 10 was performed. Table II summarizes results of analysis of the data.

An F-ratio computed on data, proved to be highly significant. That no significant differences existed between means of 3 groups that met criterion (A, B, and D) is borne out by a t-test of the difference between means of Groups A and the poorer of the 2 irradiated groups that met criterion (*i.e.*, Group D). Since the learning problem involved discriminating between bright and dim light, the possibility arose that a poor performance might reflect not impaired capacity to learn but rather impaired visual function. The latter is particularly applicable to offspring of Group II since microphthalmia and anophthalmia occurred in approximately 40% of animals in this group and it is possible that ocular defects were present in Group B which were not apparent by gross inspection. Since these rats attained criterion however, on the same day as animals in Group D and only a day after the offspring of non-irradiated controls

(Group A), it is apparent that any ocular defects that may have been present in rats in this group were not sufficiently marked to impair their learning the discrimination problem. The slightly poorer performance of rats in Group B during the early phase of learning (Fig. 1) as compared to that of rats in Groups A and D may have been due to such defects. Additional evidence that the learning performance of rats in Group B was not genuinely lower than that of Group D is indicated by the error score of rats in the 2 groups on day 10 (Table I).

Present findings indicate that prenatal x-irradiation significantly altered behavioral performance of rats exposed at 6 months of age to a brightness discrimination problem. The effects obtained were dependent on stage of pregnancy when x-irradiation was administered and dosage employed. After a single dose of 150 r, discrimination learning performance was impaired only in offspring of rats irradiated on 14th day of pregnancy. After a dose of 300 r, discrimination learning performance was impaired in offspring of rats irradiated on 18th day of pregnancy as well. These observations are in accord with previous findings that prenatal x-irradiation impaired the maze learning performance of rats (2,4,5) and provide additional evidence that sublethal doses of x-irradiation during the latter trimester of pregnancy may result in a learning decrement in the rat.

Summary. Offspring of non-irradiated rats which received a single dose of 150 r x-irradiation on 10th, 14th or 18th day of pregnancy respectively or 300 r on 18th day of pregnancy were exposed at approximately 6 months of age to a brightness discrimination problem. Data were evaluated on the basis of number of trials to criterion and number of errors for the various groups on the day when offspring of non-irradiated group reached criterion. Findings indicate that a single exposure of 150 r on 14th day or 300 r on 18th day of pregnancy significantly impaired discrimination learning performance as compared to the performance of non-irradiated rats or offspring of rats administered a single dose of 150 r x-irradiation on 10th or 18th day of pregnancy.

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Inhibition of Adrenal Medullary Responses to Chemical Stimulation.* (24531)

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Hexamethonium antagonizes the ganglionic stimulation produced by acetylcholine and other depolarizing substances such as nicotine or tetramethylammonium(1). The adrenal medulla is in many ways analagous to a sympathetic ganglion and is known to respond to ganglionic stimulating agents by release of medullary hormones(2). However, the influence of ganglionic blocking agents on responsiveness of adrenals to direct chemical stimulation has not been adequately described. In this study, experiments have been carried out to determine effects of hexamethonium and of

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† Medical Student Fellows, Foundation for Infantile Paralysis.

a newly synthesized substituted urea, N-N-diisopropyl - N′-n-butyl-N′-diethylaminoethyl urea (P-275, Pitman-Moore‡), on responsiveness to the well known chromaffin cell stimulants, nicotine and potassium.

Method. Injection of small quantities of nicotine or potassium chloride into the thoracic aorta has been shown previously to produce marked increments in circulatory catechol amines(3) and in cardiovascular functions(4). These responses are thought to be due to direct stimulation of the adrenal medullae since they are not obtained by intravenous injection of equal doses of these agents or by intra-aortic injection in adrenalectomized animals(4). Adrenal stimulation was evaluated in 2 ways: a) by auto-assay technic consisting of recording changes in heart contractile force and arterial blood pressure; b) by fluorimetric determinations of concentrations of epinephrine and norepinephrine in peripheral blood plasma. Experiments were carried out in 14 open-chest, bilaterally vagotomized dogs anesthetised with intravenous sodium pentobarbital (30 mg/kg). The chest was opened by mid-line incision and a strain gauge arch(5,6) sutured to right ventricular muscle for measurement of heart contractile force. Blood pressure was obtained with a Statham transducer from a catheter in left femoral artery. This catheter was also used to obtain blood samples for the plasma catechol amine determinations, using a modification(7) of method described by Weil-Malherbe and Bone(8). Intra-aortic injections were made by means of a polyethylene catheter introduced through right femoral artery, into the thoracic aorta, to level of apex of the heart.

Results. Fig. 1 shows the typical marked contractile force increments produced by intra-aortic injections of 10 μg/kg of nicotine and 2 mg/kg of potassium chloride. After administration of 5 mg/kg of hexamethonium intravenously, the heart force response to nicotine is substantially less than the control response, while the effects of potassium are essentially unchanged (Fig. 1).

Table I summarizes mean percentage

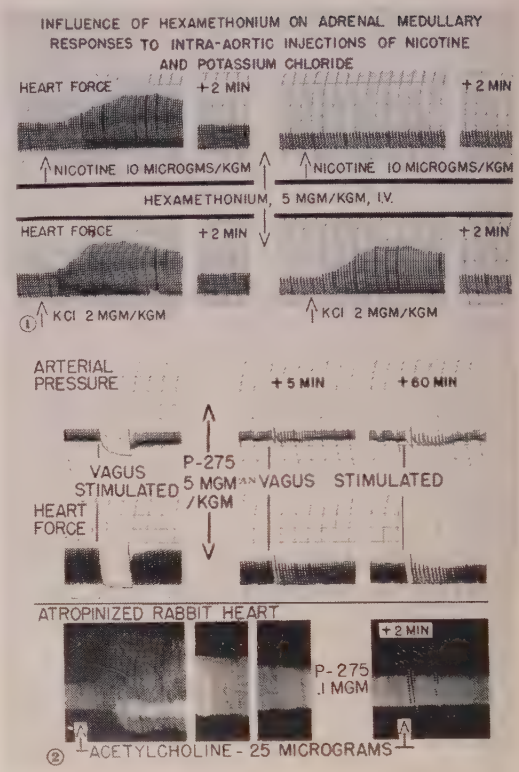

FIG. 1.

changes in heart force and blood pressure obtained in 5 similar experiments, and presents simultaneous variations in plasma levels of epinephrine and norepinephrine. Epinephrine and norepinephrine are presented as mean increases above control values, expressed in μg/liter of plasma. With nicotine, increments in heart force, blood pressure and plasma epinephrine obtained in the control, or pre-block animals were not obtained after hexamethonium (Table I). In contrast to the lack of responses to nicotine after hexamethonium, the mean responses to potassium appeared to be augmented by administration of the ganglionic blocking agent (Table I). These differences were not statistically significant ($P > .10$).

The effects of compound P-275 on adrenal responsiveness to nicotine and potassium were qualitatively similar to those produced by hexamethonium. Intravenous injection of 5 mg/kg of P-275 substantially reduced the increments in contractile force, blood pressure

‡ Supplied through courtesy of Pitman-Moore Co., Indianapolis, Ind.

TABLE I. Influence of Hexamethonium and P-275 on Adrenal Stimulations Produced by Nicotine and Potassium.

	Nicotine, 10 μg/kg			Potassium chloride, 2 mg/kg		
	Pre-block	After hexamethonium	After P-275	Pre-block	After hexamethonium	After P-275
No. observations	14 (14)†	5 (5)	11 (8)	14 (12)	5 (5)	10 (6)
	Maximum % increase above control (mean ± S.E.)					
Heart force	140 ± 15	0 ± 0*	57 ± 19*	125 ± 17	161 ± 67	103 ± 22
Blood pressure	57 ± 11	5 ± 3*	13 ± 5*	40 ± 4	80 ± 23	24 ± 8
	Increase above control in μg/l of plasma (mean ± S.E.)					
Epinephrine	15.9 ± 3	.2 ± .2*	3.0*	12.0 ± 3.9	23.0 ± 8.0	8.3 ± 2.1
Norepinephrine	2.1 ± 1	.2 ± .4	.5	4.0 ± 1.9	5.8 ± 1.7	1.9 ± .4

* Significantly different from Pre-block responses ($P < .05$). † No. of dogs in parentheses.

and plasma epinephrine produced by nicotine, but did not alter responsiveness to potassium (Table I).

The basal levels of catechol amines were not significantly influenced by hexamethonium or P-275. The following are the mean values (± SE) for epinephrine and norepinephrine respectively, expressed as μg/liter of plasma: a) before ganglionic blockade, 0.7 ± 0.2 and 1.7 ± 0.3; b) after hexamethonium, 0.4 ± 0.1 and 2.0 ± 0.5; c) after P-275, 0.6 ± 0.1 and 1.4 ± 0.3.

In addition to its adrenal blocking activity, P-275 was found to have other properties in common with hexamethonium. In 4 experiments, arterial pressure and heart force were recorded in dogs during electrical stimulation of the peripheral end of cut vagus nerve. In the untreated control, vagal stimulation of 5 seconds duration produced cardiac standstill (Fig. 2). Approximately 5 minutes after administration of P-275 (5 mg/kg) similar stimulation provoked only a minimal decrease in heart rate. After one hour, brief cardiac arrest could again be produced, and generally about 2 hours was required before a normal response could again be obtained. P-275 did not alter responsiveness to injected acetylcholine, indicating that the blockade must occur at the ganglionic synapse.

The lower tracing of Fig. 2 illustrates the stimulant effect of a large dose of acetylcholine on the isolated atropinized rabbit heart. Addition of 0.1 mg of P-275 to the perfusion solution completely abolished the response to a subsequent dose of acetylcholine. In several experiments, doses of acetylcholine as high as 200 μg were administered after P-275 and failed to produce any evidence of stimulation. Both of these actions of P-275, namely, vagal ganglionic inhibition and blockade of the acetylcholine response in the rabbit heart, were also produced by comparable doses of hexamethonium.

Discussion. Our experiments demonstrate that hexamethonium markedly reduces responsiveness of the adrenal medulla to direct stimulation with nicotine, while the effects of potassium are unchanged. Previous investigators have shown that the ganglionic excitant effects of potassium are not significantly altered by tetraethylammonium(9). These data indicate that the adrenal medulla is identical to other sympathetic ganglia in its reaction to ganglionic blocking agents(1,9). Thus it would appear that the ganglionic blocking activity of drugs could be simply and effectively estimated by determining their ability to antagonize adrenal responsiveness to direct chemical stimulation. The experiments with compound P-275 support this hypothesis.

P-275 is a newly synthesized substitute of urea which has been shown by its commercial sponsors to abolish the pressor response to large doses of acetylcholine in atropinized dogs, and to reduce the hypertensive effect of splanchnic nerve stimulation (personal communication). In the present experiments, this compound was found to be similar to hexamethonium in reducing the adrenal excitant effects of nicotine, but not of potassium. Further evidence of the ganglionic blocking activity was obtained in demonstrating that P-275, in common with hexamethonium, pro-

duced vagal ganglionic inhibition and blockade of the stimulant effect of acetylcholine on the isolated rabbit heart.

Summary. Hexamethonium reduced sensitivity of adrenal medulla to nicotine, but not to potassium. A newly synthesized compound, N-N-diisopropyl-N′-n-butyl-N′-diethylaminoethyl urea (P-275) was similar to hexamethonium in its effects on the adrenal, as well as in its ability to inhibit the cardiovascular depressant effects of vagal stimulation and the acetylcholine stimulation of the isolated atropinized rabbit heart.

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Isolation of Mucosal Disease Virus by Tissue Cultures in Mixture 199, Morgan, Morton and Parker.* (24532)

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Isolation of viruses by tissue culture methods from cattle with bovine mucosal disease, infectious bovine rhinotracheitis, and virus diarrhea of New York type of mucosal disease complex(1) have been reported by at least 4 teams of investigators. Underdahl, *et al.,*(2) isolated a cytopathogenic virus from cattle having bovine mucosal disease (BMD). York, Schwartz and Estela(3) and Madin and his associates(4) isolated viral agents from cattle having infectious bovine rhinotracheitis; Lee and Gillespie(5) isolated a strain of virus from cattle with virus diarrhea New York. All foregoing virus isolations employed trypsinized cells and medium containing serum from cattle, horse or lamb. Lee and Gillespie also used roller tube explant method isolation procedures. Underdahl (personal communication) believed that his success in isolation and cultivation of a viral agent from animals having BMD depended upon using a homologous system of bovine kidney cells and bovine serum. He obtained antibody free serum by drawing blood from a newborn calf which had not been allowed to nurse. However, Schipper and Eveleth(6) report calves being born and dying 18 to 96 hours after birth showing pathology typical to BMD upon necropsy. They also report similar pathology in calves born dead at full term. In one instance, necropsy of a mature cow diagnosed as having mucosal disease possessed an 8½ month old fetus with typical lesions of mucosal disease. No BMD virus or hyperimmune serum was demonstrated from such calves since virus isolations procedures were not yet established. Since that time however, we have had a still born full term calf which showed pathology typical to BMD. While no viruses were recovered from biopsies of this calf, the serum, when diluted 1:64, was capable of neutralizing 1000

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$TCID_{50}$ of the BMD virus. While serum has value in promoting cellular growth *in vitro* its necessity for virus propagation is questionable. Mixture 199† (7) allowed proliferation of trypsinized bovine kidney cell cultures, thus supplying cellular metabolites that might possibly be utilized by viruses in their propagation, and eliminated the variable of serum.

Methods and materials. Bovine kidney cells were obtained by trypsinization, according to Youngner (8) as modified by Madin (9). The cortex of kidneys of bovine feti approximately 3 to 4 months of age were used as tissue source. Suspension of cells was washed 3 times and diluted 1:10 with Mixture 199 following final stage of trypsinization. 0.1 ml was then seeded directly into 125 x 15 mm screw cap test tubes containing 1 ml of Mixture 199 to which 100 units of penicillin and 100 mg of streptomycin/ml had been added. The resulting cell suspension yielded 500,000 to 800.000 cells tube. The tubes were incubated at 36°C in stationary rack at 5° angle for 72 hours to allow cells to adhere to glass, during which time a substantial monolayer was formed. At the end of 72 hours the medium was replaced with 1 ml of fresh Mixture 199 and 0.1 ml of inoculum was added to half of the tubes. The BMD inoculum was prepared from 3 tissue sources of animals displaying symptoms prior to death and pathology typical of mucosal disease upon subsequent necropsy. One inoculum was derived from mesenteric lymph nodes, one from spleen tissue and the third inoculum was pooled tissues containing lymph nodes, spleen and whole blood. All the inoculum was processed by diluting 1:10 with Hank's solution and homogenized in a Virtis tissue homogenizer. Following centrifuging at 3000 rpm for 20 minutes the supernatant was diluted 1:100 with Hank's solution and treated with 1000 units of penicillin and 1000 μg of streptomycin/ml for one hour under refrigeration. This material was then used as tissue culture inoculum or stored at –40°C.

Results. 1. *Isolation and cytopathogenic effects (CPE) of virus.* CPE was noted in tubes containing both spleen and pooled tissue inoculum but not in tubes containing lymph node inoculum. The CPE on trypsinized bovine kidney cells consisted primarily of vacuolation. Small vacuoles appeared in the cytoplasm approximately 4 to 6 days following inoculation. Vacuoles increased in size and terminated with destruction of the cells which resulted in open patches in tissue cultures until eventually the tubes were virtually devoid of cells. Subpassages of fluid from the tubes showing CPE regularly reproduced these characteristics in tubes containing trypsinized bovine kidney cells and the CPE required approximately the same amount of time to make its appearance in such subpassages. No CPE was noted in repeated passages to tubes containing the following cell lines: HeLa cells, trypsinized embryonic bovine testicular cells, embryonic bovine skin-muscle roller tube explants, or trypsinized embryonic ovine kidney cells. Negative results were also noted from repeated passage in the following embryonic animal kidney and skin-muscle roller tube explants: rabbit, mouse, hamster and chick fibroblast. Control tubes without added inoculum were maintained in Mixture 199 for over 15 days without changing Mixture 199 or other cell feeding, and displayed no apparent cellular deterioration. To determine whether bovine tissue inoculum would in itself have CPE on trypsinized bovine kidney cells, inoculum control tubes were established. Three inocula were prepared as previously described from a calf with acute Salmonellosis upon necropsy. This calf was negative for BMD as based on the serum neutralization test. In such control tubes the inoculum had no CPE on the bovine kidney cells in either initial tubes or subsequent subpassages.

2. *Pathogenicity of tissue culture virus for calves.* Two 8-month-old Holstein calves, Nos. 976 and 979, found negative for neutralizing antibodies for BMD as based on serum neutralization test, were injected intravenously with 2 ml of tissue culture virus from the 3rd passage. Both animals were then housed in a heated, well ventilated barn for observation. A blood specimen was taken

† Mixture 199 was supplied by Microbiological Associates, Washington, D.C.

TABLE I. Neutralization of Tissue Culture BMD Virus by Antisera from Various Sources.

Serum source	Inoculum	Homologous serum dilution neutralizing 1000 $TCID_{50}$* Pre-inoculation	Convalescent
	Tissue culture virus		
Calf 976	7–799A	0	1:128
979	"	0	"
Rabbit A–31	"	0	1:64
32	8–373A	0	"
33	8–261A	0	1:32
34	8–379A	0	1:64

* All heterologous cross neutralizations were demonstrated at the same dilutions as homologous neutralization titers.

from each animal every fifth day for 2 months. From 6 to 10 days following virus injection the calves had mild temperature rises and one, No. 976, suffered a 2-day period of mild diarrhea. From 15 to 25 days post-inoculation the calves blood demonstrated neutralization antibodies for BMD virus. Absence of the more serious manifestation of bovine mucosal disease as observed in feed lot conditions, may be explained by sheltering of experimental animals from natural environmental stress factor.

3. *Serum neutralization studies.* Serum from above animals was mixed with equal volumes of tissue culture virus fluids in final concentrations of 1000 $TCID_{50}$ virus. Serum-virus mixtures were incubated at 20°C for 2 hours and 0.8 ml was inoculated into each of 10 tubes. Tests were determined when cells in the virus control tubes showed complete CPE.

Convalescent serum from calves that had been injected with virus from the 3rd tissue culture passage, diluted 1:128, neutralized 1000 $TCID_{50}$ of virus, while undiluted pre-inoculation serum from the same calves failed to neutralize the virus.

The relationship of viruses isolated from cattle having symptoms and pathology of BMD in 4 widely separated areas of North Dakota was demonstrated with serological studies conducted with rabbits. Two rabbits were injected twice intravenously with 1 ml of 8th passage tissue culture virus from each of the 4 original BMD virus isolates. Rabbits were bled 10 days before the first virus injection and 20 days following last injection. None of the pre-inoculation serum demonstrated neutralizing antibodies. However, convalescent serum when diluted 1:64 in 3 cases and 1:32 in one instance was capable of neutralizing 1000 $TCID_{50}$ of virus. In cross neutralization studies, hyperimmune serum from any of the test rabbits was capable of neutralization of all of the 4 originally isolated BMD viruses. The results of the serum neutralization studies are presented in Table I.

Summary. A virus has been isolated from cattle which displays typical symptoms and pathology of bovine mucosal disease. Virus isolations and propagation were carried on using tissue cultures of trypsinized bovine kidney cells in Mixture 199 exclusively. Two calves which were negative for neutralizing antibodies for bovine mucosal disease were injected with tissue culture virus and developed hyperimmune serum from 15 to 25 days post-inoculation. Serology conducted with laboratory rabbits indicate similarity of viruses isolated from 4 separate herds of cattle in North Dakota.

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Detection and Possible Source of Contaminating Pleuropneumonialike Organisms (PPLO) in Cultures of Tissue Cells. (24533)

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One line of conjunctival cells and 3 of 5 lines of HeLa cells were reported by Robinson *et al.*(1) to be contaminated with pleuropneumonialike organisms (PPLO) even though the cultures routinely contained 100 units of penicillin and 100 μg of streptomycin /ml of medium. Collier(2) found 11 of 14 lines of HeLa cells contaminated with PPLO. The 3 lines which were not contaminated had at some time been cultivated in the presence of aureomycin, terramycin or neomycin. This report deals with research directed towards determining the extent of PPLO contamination of tissue cell cultures and source of this contamination.

Materials and methods. Samples of tissue cell cultures were obtained from 5 laboratories as listed in Table I. Multiple samples containing both cells and supernatant were taken at varying periods during growth of the tissue cells. The tissue cells were grown in Hank's, Eagle's or medium 199 containing 10 to 20% horse, human, calf, rabbit or dog serum. One ml of tissue cell culture containing both cells and supernatant was transferred to 5 ml of Bacto-PPLO broth(3) enriched with 1% PPLO serum fraction(4). After 3 days aerobic incubation at 37°C, 0.2 to 0.4 ml of this culture were inoculated over the entire surface of Bacto-PPLO agar(3) enriched with 1% PPLO serum fraction. Plate cultures were incubated aerobically at 37°C. In some cases duplicate tests were made by inoculating tissue cell cultures directly onto the agar medium. From the third to seventh day the inverted Petri dish cultures were examined by transmitted light under low power of microscope (100 ×). Presence of PPLO colonies was established by attempting to move the colonies with a wire loop, staining with Dienes stain, and repeated sub-culture on agar medium. Samples of both fresh and frozen horse, human, and calf serum were obtained from stock supplies used at the various laboratories. Both individual and pooled samples were tested for PPLO by inoculating 1 ml of sample into 5 ml of PPLO broth enriched with 1% PPLO serum fraction. These cultures were incubated 3 days at 37°C then 1 ml was transferred to a second tube of same medium. After 3 more days incubation the broth cultures were plated, incubated, and examined as previously described for testing of tissue cell cultures.

Results. Colonies of PPLO appeared on the surface of agar medium 3 to 5 days after inoculation. The colonies, upon primary isolation, varied considerably in size and morphology. The diameters of colonies ranged from 0.08 mm to nearly 0.3 mm. The morphology varied from vacuolated, lacy, to small dense refractile types. After repeated passage on agar medium the colonies assumed the classical fried egg appearance (Fig. 1). Colonies stained with Dienes stain and would not rub off the surface of the agar. All PPLO contaminants which were isolated required serum or serum fraction for growth.

Direct seeding of tissue cell cultures onto the agar medium did not increase the frequency of positive isolations. PPLO colonies, which were not as numerous as after one passage in broth, were more difficult to recognize among the debris of tissue cells.

Frequently strains of PPLO did not grow well until after 3 or 4 serial transfers on agar medium. Occasionally, when a tissue cell line had been cultured repeatedly, a negative result was obtained among a series of positive isolations. This probably reflects unavoidable faults in sampling or cultivation.

Of 37 cell lines tested, 22 were contaminated with PPLO. Cell lines derived from human, monkey, and mouse tissues were positive. All cells in first or second passage were negative. PPLO were isolated from cells growing in human, horse, and calf sera. All but 3 positive cell lines had at some time been grown in presence of human serum. Two lines of monkey kidney cells and a line

TABLE I. Cell Cultures Examined for Presence of Pleuropneumonialike Organisms.

Cells	Type	Source*	Serum	Passage	Lines + / lines tested
HeLa	human carcinoma	1	human	40– 64	1/1
"	" "	2	" & calf	25–121	7/10
"	" "	3	" horse		1/1
H. Conj.	" conjunctiva	1	" "	88–100	"
H. K.	" kidney	1	" "	99–111	"
C_3	" fibroma	1	"	11	"
Int.	" embryonic intestine	1	"	40– 48	"
KB	oral cancer	2	"	22	"
Lung	human lung	3	" & horse		"
MCN	" bone marrow	3	" "		"
Hep_2	" epithelioma	1	"	10	"
Hep_1	" "	2	"	15	0/1
Am	" amnion	2	" & calf	20	1/2
Chang L	" liver	2	"	11	1/1
MK	monkey kidney	4	horse	100–200	2/2
"	" "	1	calf	1	0/1
"	" "	3	human	1	"
SCH	" heart	1	calf	70	"
L	mouse mesenchymal	4	horse	100	1/1
ERK	embryonic rabbit kidney	1	rabbit		0/1
RBK	adult " "	1	calf		"
RK	*Idem*	1	human & calf	1– 2	"
"	"	3	calf	1	"
BK	bovine kidney	1	"		"
PK	porcine "	1	"		"
Dog	dog fascia	5	dog	22	"

* 1 = So. Jersey Med. Research Foundation, Camden, N. J.
2 = Univ. of Miami School of Med.
3 = Children's Hosp., Philadelphia.
4 = Connaught Medical Research Labs., Univ. of Toronto.
5 = Presbyterian Hosp., Philadelphia.

of mouse mesenchymal cells, which were never in contact with human serum, were contaminated with PPLO.

None of the contaminated cell lines demonstrated gross cytopathogenic effects and no turbidity was evident. In some lines it was noted that the infected cells failed to adhere to the glass surface in continuous sheets.

All 25 samples of sera tested were negative for PPLO. These included 7 pooled lots of horse serum, 2 pooled samples of calf serum and 16 samples of human serum comprising pooled lots and individual samples.

Discussion. Extensive contamination of tissue cell cultures by PPLO could have profound effects on research in which tissue cells are being studied or used as tools. Presence of PPLO in tissue cell cultures has been shown by Coriell *et al.*(5) to account for some serological cross reactions between different lines of tissue cells. The effects of PPLO contamination on cell metabolism and viral growth in cells remains to be studied.

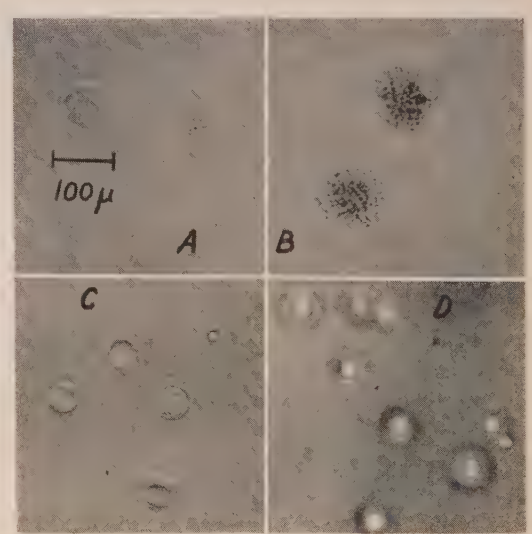

FIG. 1. A. Vacuolated type of colony of PPLO on first passage on solid medium from oral cancer cell line. B. Lacy type of colony in same culture as A. C. Dense type of colony from strain of PPLO isolated from HeLa cells. D. Fried egg appearance of PPLO colonies in same culture as C. All cultures were 8 days old when photographed. 80×.

Failure of tissue culture PPLO to cause cytopathogenic effects or turbidity makes culturing a necessity. Shepard(6) and Wittler *et al.*(7) reported intracellular growth of human genital strains of PPLO and Nelson(8) demonstrated intracellular growth of mouse strains of PPLO. Preliminary observations indicate that this is also true in the case of PPLO contaminants of tissue cell cultures. This feature should be kept in mind when attempting to free tissue cell cultures of their contaminating PPLO.

The source of the contaminating PPLO is unknown. It was suggested by Robinson *et al.*(1) that presence of PPLO in tissue cell cultures was probably due to contamination of some common component of culture media. Although most contaminated cell lines have been in contact with human serum, 3 positive isolations have been made from cells grown in horse serum and which had never been in contact with human serum. These isolations together with failure to isolate PPLO from samples of human serum as reported here, and previous failures to isolate PPLO from routine blood cultures(9,10) indicates that serum is probably not the source of the contaminants.

Collier(2) suggested that the cell lines were contaminated with PPLO when originally isolated. This hypothesis could explain contamination of some cell lines, for PPLO are frequently isolated from human genitourinary tract(9) and oral cavity(11,12). This suggestion, however, would not explain the presence of PPLO in strains of cells derived from such sources as human bone marrow and liver, monkey kidney and mouse mesenchymal tissues, or for greater frequency of contamination in cell lines which have undergone numerous passages.

Direct introduction into tissue cell cultures of human PPLO by a break in aseptic technic could account for the PPLO contamination. Serological comparisons now under way may help to clarify the role of human strains of PPLO in contamination of tissue cell cultures.

It is unlikely that the PPLO were introduced into tissue cell lines during testing, for the contaminants have not serologically reacted, as far as tested, with our stock laboratory strains of PPLO.

Another possible source of contamination is by formation of stabilized L forms of contaminating bacteria by action of penicillin which is widely used in tissue cell cultures. Formation of stable L forms has been demonstrated with *Streptococci*(13), diphtheroids (14), *Streptobacillus moniliformis*(15), *Proteus*(16), and *Salmonella*(17). Reversion of a human genital PPLO to a *Corynebacterium* has been reported(7) during tissue culture passage and conversion of a stock strain of human genital PPLO to a diphtheroid has been demonstrated(18). Minck(19) reported that 5 strains of PPLO isolated from female genital tract developed into diphtheroids and McKay and Taylor(20) described reversion of poultry strains of PPLO to bacteria similar to *Hemophilus gallinarum*. By definition, the difference between L forms of bacteria and PPLO is that L forms are known to be associated with some bacterial species while PPLO are not. Bacteria are common contaminants in tissue cell cultures and it is possible that the PPLO contaminants represent stable L forms produced from the contaminating bacteria by the action of penicillin.

Summary. 1) A total of 37 tissue cell lines growing in the presence of horse, calf, human, rabbit or dog serum have been tested for contamination by PPLO. Of these, 22 or 59% contained PPLO. Human, monkey, and mouse cell lines were contaminated. All first and second passages of cell lines were negative. PPLO were isolated from tissue cells growing in human, horse or calf serum. Nineteen positive cell lines were, at some time, grown in presence of human serum. Three positive lines growing in horse serum had never been in contact with human serum. 2) All 25 samples of sera, including pooled lots of human, calf, and horse sera, were negative for PPLO. 3) Possible sources of PPLO contamination of tissue cell cultures are discussed.

We wish to express our appreciation to Dr. Lewis L. Coriell (S. Jersey Med. Research Fn.), Dr. Werner Henle (Children's Hosp., Philadelphia), Dr. M. Michael Sigel (U. of Miami), Dr. Ralph B. Gwatkin (Connaught Medical Research Labs.), and Dr. Angelina Frabrizio (Presbyterian Hosp.) for their interest and samples of tissue cell cultures.

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Effect of Insulin on Glucose Uptake by Mouse Diaphragm Tissue. (24534)

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The effect of insulin in increasing glucose uptake by isolated rat diaphragm tissue has been employed by various workers(1-7) for estimating minute amounts of insulin. Our preliminary results in determining insulin activity using the rat hemidiaphragm technic were so often variable that we undertook the present study to develop a more reliable test method. We believe the method to be described employing pooled mouse hemidiaphragms is an improvement over the rat diaphragm technic from the standpoint of sensitivity and reproducibility. In developing the method, a systematic study was made of the various factors affecting glucose uptake to assess their effect upon the assay of insulin.

Materials and methods. Swiss-Webster and NIH general purpose male albino mice were used in most of our studies. A few experiments were made with DBA-2 and C-57 strains of mice. No difference in insulin responsiveness which could be attributable to strain differences was found. For each experiment the mice were of same weight ($\pm$ 2 g) and in most experiments were in the 25-30 g weight range. Mice were fasted for 16-24 hours before use. Unless otherwise stated, the medium employed for incubation was a modified Krebs-Ringer-bicarbonate solution, hereafter referred to as KRB solution, having the following composition in millimoles/l: NaCl (95), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), and $NaHCO_3$ (50). Glucose was added to give a concentration of 1 mg/ml. The insulin used was a glucagon-free, crystalline zinc sample (Eli Lilly & Co.) which assayed 24.6 units/mg. Dilutions of a stock insulin solution (10 units/ml) were made before each experiment with 0.9% saline solution which had been brought to pH 3 with 1 N HCl. A systematic procedure was followed in preparation of the diaphragms. Groups of 5 mice were treated successively in the following manner. As each mouse was killed by decapitation its diaphragm was excised with a pair of iris scissors and placed in ice cold KRB solution (without glucose) which was gassed continuously with a mixture of 95% O_2-5% CO_2.

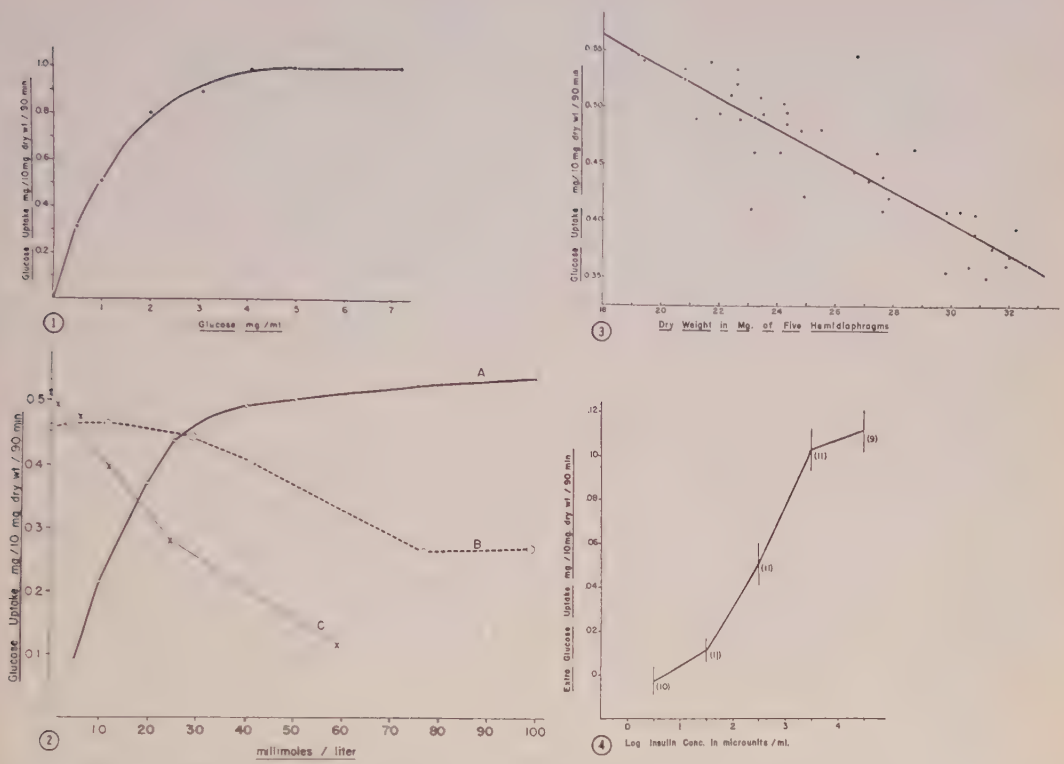

FIG. 1. Effect of glucose concentration on uptake. Each point represents uptake of 5 pooled mouse hemidiaphragms incubated in 3 ml of KRB medium.

FIG. 2. Effect of bicarbonate (A), potassium (B), and magnesium (C) concentration on glucose uptake.

FIG. 3. Effect of diaphragm size on glucose uptake.

FIG. 4. Increase in glucose uptake as a function of insulin concentration. No. of experiments in parentheses; stand. errors as vertical bars.

When all 5 diaphragms had been removed, they were rinsed with fresh medium and each diaphragm was cut in half after careful removal of adipose tissue and the thick posterior portion. The hemidiaphragms were blotted on filter paper and transferred to chilled Warburg vessels containing appropriate incubation medium. Each test vessel contained 5 hemidiaphragms from 5 mice and a second Warburg vessel containing the other hemidiaphragms from the same mice served as a control. For assay of insulin each test vessel contained 3 ml of KRB (1 mg glucose/ml) and 0.10 ml of the appropriate insulin dilution; each control vessel contained 3 ml of KRB glucose solution and 0.10 ml of insulin diluent. The flasks were placed in 37°C bath, gassed with 95% O_2-5% CO_2 for 10 minutes and incubated for 90 minutes at a shaking rate of 130 cycles/min. Approximately 15 minutes were required for preparation of the diaphragms of each group. A single experiment usually consisted of 6 groups (30 mice). Glucose remaining in the medium after 90-minute incubation was determined by the Somogyi modification of Nelson's method(8) using zinc sulfate-barium hydroxide filtrates. The diaphragms from each flask were heated at 110°C for 2 hours to obtain dry weights and glucose uptake calculated as mg glucose taken up from the medium/10 mg dry weight of diaphragm tissue.

Results. Effect of glucose concentration. Fig. 1 shows the effect of varying glucose concentration upon uptake of glucose by pooled mouse hemidiaphragms. Test hemidiaphragm pools were incubated in 3 ml of KRB solutions of varying glucose concentrations and each of the corresponding control pools were incubated in 3 ml of KRB solution containing

1 mg glucose/ml. The uptake was calculated for each glucose concentration as follows: $\frac{t}{c} \times \bar{c}$ = calculated uptake in which t = observed uptake of test, c = observed uptake of corresponding control, and $\bar{c}$ = average uptake of all controls. The uptake was markedly increased as glucose concentration was raised from 0.5 to 2 mg/ml; however, with higher concentrations the uptake leveled off, indicating an approach to maximal rate. Since glucose concentration falls off progressively during incubation, uptake rate progressively decreases and the values shown represent overall average rates for 90-minute incubation period. Under conditions selected for assay of insulin, initial glucose concentration of 1 mg/ml fell to approximately 0.6 mg/ml during incubation. The insulin effect was more reproducible under these conditions than with higher glucose concentrations although the absolute insulin effect was smaller.

Effect of ionic composition. The effect of bicarbonate, potassium, and magnesium concentrations in KRB solution upon basal glucose uptake is shown in Fig. 2. Uptake values were calculated according to the method described for effect of glucose concentration on uptake. The isotonicity of the modified KRB solutions was maintained by adjusting NaCl concentration. The profound effect of bicarbonate concentration (Curve A) on uptake was a pH effect since under a constant CO_2 pressure, the pH of the HCO_3-CO_2 buffer system varies with the HCO_3^- concentration. HCO_3^- concentration range of 5 to 100 millimoles/l corresponds to pH range of 6.2 to 8.2(9). To minimize the effect of any pH change upon uptake during incubation, a 50 millimolar bicarbonate concentration (pH 7.9) was selected for assay of insulin. A significant reduction in uptake rate was effected when potassium concentration exceeded 40 millimolar (Curve B). These results are similar to those of Stadie and Zapp(10) who found that glycogen synthesis by the rat diaphragm was markedly reduced by high potassium concentrations. Due to progressively stronger inhibitory influence of magnesium ion on basal uptake (Curve C), this ion was omitted from the assay medium. Studies on the effect of calcium were limited because Ca CO_3 precipitated from the KRB medium when calcium concentration exceeded 5 millimolar. In the absence of calcium the uptake was increased by an average of about 6% compared to the uptake in KRB medium with calcium. Diaphragms incubated in an isotonic medium having sodium as its only cation constituent (105 millimolar NaCl, 50 millimolar $NaHCO_3$) showed an average 20% lower uptake than diaphragms incubated in KRB medium. Moreover, diaphragms incubated in saline-HCO_3 medium became significantly hydrated and showed total water content of 2-3% more than the average of 78.1% for diaphragms incubated in the KRB medium.

Effect of diaphragm size. It was observed that basal uptake rates of diaphragms from large sized mice were lower than those of smaller sized mice. This inverse relationship between uptake and diaphragm size is shown by Fig. 3. The linear regression line as calculated by the method of least squares is: $Y = -0.135X + 0.805$, where Y is the basal uptake in mg of glucose/10 mg dry wt/90 min and X is the dry weight in mg of 5 pooled hemidiaphragms. The data were obtained from mice weighing 18 to 30 g. Under comparable conditions, the basal uptake of mouse diaphragms was 2½ to 4 times greater than the uptake of diaphragms from rats weighing approximately 100 g. Although this difference might be due to a higher specific metabolic activity of the mouse tissue, the effect of the larger surface exposure for the same weight of tissue must be considered. Rat hemidiaphragms which were cut into smaller pieces consistently showed a higher glucose uptake; for example, when the hemidiaphragms were quartered the uptake was increased by an average of 9%. With mouse hemidiaphragms, on the other hand, similar treatment reduced the uptake by an average of 6%. These results indicated to us that with rat diaphragm tissue the cellular damage caused by mincing was more than compensated by an increase in surface exposure resulting in net increase in uptake. On the other hand, with mouse tissue in which a

thinner dimension favors near maximal uptake rates, the cellular damage produced by mincing overcomes any effect of an increase in surface area.

Effect of insulin. The extra amount of glucose taken up by pooled hemidiaphragms incubated in presence of varying concentrations of insulin is shown by Fig. 4. The extra glucose represents the difference in uptake of insulin-treated hemidiaphragm pool and its corresponding control pool. A linear regression line was obtained between 1.5 and 3.5 log micro-units insulin/ml. The data represent results of 11 consecutive experiments and include all values to illustrate degree of variability and of reliability of the assay under routine conditions ($\lambda = 0.59$). In contrast to our experience with the rat hemidiaphragm technic, where on occasion a hemidiaphragm would not respond to insulin, an insulin response was consistently elicited by the pooled mouse diaphragm technic. It must be emphasized, however, that since the slope of the regression line varied significantly from experiment to experiment, it is necessary to include insulin standards whenever test samples are run. The minimal detectable concentration by this method was approximately 30 microunits of insulin per ml.

Discussion. One of the problems encountered using the rat hemidiaphragm technic has been the variability of individual hemidiaphragms to the action of insulin. Various modifications have been used to minimize this factor such as using pooled hemi-, quarter-, and fifth-diaphragms(3,11,12). Mouse diaphragm tissue because of its smaller size and greater glucose uptake ability is particularly suited for this pooling procedure for, on a weight basis, more than 4 mouse diaphragms can be substituted for a single rat diaphragm.

A further advantage in using mouse diaphragm tissue is its higher surface to mass ratio with a greater proportion of cells in direct contact with the incubation medium.

It is difficult to compare relative sensitivity of mouse diaphragm technic to rat diaphragm technic since the minimum detectable amount reported for the latter method varies over a considerable range(6). Comparative experiments in our laboratory have shown the mouse diaphragm technic to be superior both in terms of sensitivity and reproducibility. Further work is now in progress to apply this method to determination of insulin activity in blood.

Summary. A method for estimating insulin using pooled mouse hemidiaphragms has been developed. A linear log dose-response was obtained between 30-3000 micro-units of insulin/ml. Basal glucose uptake of mouse diaphragm tissue was 2½ to 4 times that of rat diaphragm tissue. Basal uptake was significantly affected by pH of medium, glucose concentration, and diaphragm size. A progressive increase in magnesium or potassium concentration of the medium suppressed the basal glucose uptake.

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Correlation between Granulation of Juxtaglomerular Cells and Extractable Renin in Rats with Experimental Hypertension.* (24535)

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Twenty years ago, Goormaghtigh theorized without much evidence that granules in the juxtaglomerular cells contained renin(1). This concept was strengthened somewhat when Peart, Gordon, Cook and Pickering reported that renin in rabbit kidney was present only in areas containing glomeruli and not in zones which contained only tubules(2). Recently, the Hartrofts have developed a superb stain for juxtaglomerular granules which permits a fairly accurate quantitation of them (3). It seemed of interest to determine both juxtaglomerular granulation and renin content in the same kidney of certain experimental rats. If the 2 parameters varied together, it would strengthen Goormaghtigh's old theory. If they did not, it would militate against its validity.

Methods. The Hartroft stain for juxtaglomerular granules was utilized as previously described(3,4). Four separate sections were examined for each kidney, and the average of these 4 sections provided the "juxtaglomerular index"(3,4). For renin assay, the rat kidney was homogenized in cold isotonic saline (5 cc of saline/g of kidney) in a Potter-Elvehjem homogenizer for 8-10 minutes. Then the homogenate was shaken at room temperature for 2 hours to allow time for extraction of renin. The homogenate was then centrifuged at 3000 rpm while being maintained at 20°C for one hour. The supernatant extract was obtained for subsequent injection into "bioassay" animals weighing approximately 250 g. Rats for the bioassay were anesthetized with nembutal and nitrous oxide and the femoral artery was cannulated. The cannula and its tubing were connected by saline to a mercury manometer. 0.3 cc of the supernatant fluid from kidney homogenate was injected intraarterially in the bioassay procedure. If renin was present in the extract, a sharp rise in blood pressure occurred, reaching a maximum in 3 minutes. Blood pressure would then gradually fall to the baseline over the next 30-50 minutes. After one injection, the rats would become variably tachyphylactic to subsequent doses of extract. For this reason, all rats used to bioassay renin were not given more than one injection of the extract. The difference in blood pressure readings just before and 3 minutes after injection of the extract gives an estimate of the amount of renin contained in the extract. In *Exp. I,* a silver clip was applied to one renal artery to partially narrow its lumen. The rats were usually quite hypertensive 8 weeks after application of the clip. Renin assays on both "clipped" and the "untouched" kidneys were carried out 6 months after the original clipping procedure. In Floyer's useful terminology, the "clipped" kidney has a clip on its renal artery(5). The "untouched" kidney, which has never been surgically manipulated, is contralateral to the "clipped" kidney. Holtzman rats were used for clipping as well as for bioassay of kidney extracts. Normal blood pressure of the Holtzman strain averages 121 mm Hg (S.D. $= \pm$ 13) using the Friedman microphonic method(6) with light ether anesthesia. In *Exp. II,* desoxycorticosterone hypertension was produced by injecting 125 mg of desoxycorticosterone trimethylacetate subcutaneously and repeating this dose after a 4 week interval. Rats were allowed to drink only a 1% NaCl solution while the steroid was being absorbed. The rats were usually hypertensive by the sixth week of steroid administration, at which time renin assays on their kidneys were performed. The extract was bioassayed simultaneously on 2 test rats

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TABLE I. Granulation of Juxtaglomerular Cells and Renin Content of "Clipped" and "Untouched" Kidneys in 9 Rats with Unilateral Renal Hypertension.

	Final B.P. before obtaining kidney (mm Hg)	"Clipped" kidney JG index	B.P. response to extract from "clipped" kidney (mm Hg)	"Untouched" kidney JG index	B.P. response to extract from "untouched" kidney (mm Hg)
	146	18	+50	0	+1
	162	57	+44	2.5	−2
	156	31	+38	4	−6
	162	54	+57	2	0
	154	37	+40	1.5	−4
	164	50	+42	3.5	+1
	138	26	+40	11	+4
	174	39	+54	10	+7
	138	58	+38	7	+7
Mean	155	41	+45	4.6	+1

instead of one, and the results in the 2 rats were averaged. Kidneys from 6 normal rats served as controls. Only rats of the Wistar strain were used in this experiment. This includes rats receiving desoxycorticosterone, normal control rats, and rats receiving kidney extract in the bioassay procedure. The blood pressure of normal Wistar rats averages 102 mm Hg (S.D. = ±8). The rats in the 2 experiments were of 2 different strains with differing juxtaglomerular indices. Therefore the normal rats in *Exp. II* cannot be validly compared with the rats in *Exp. I*.

Results. Exp. I. The "clipped" kidney was compared with the "untouched" kidney in 9 rats (Table I). This was believed to be an interesting comparison since "clipped" kidneys tend to increase their juxtaglomerular granulation, while granulation in "untouched" kidneys becomes drastically reduced(4). "Clipped" kidneys had a mean juxtaglomerular (JG) index of 41 and a mean renin response of +45 mm Hg. The "untouched" kidneys had a mean JG index of 4.6 and a mean renin response of +1 mm Hg. Among the "untouched" kidneys, 3 had relatively high juxtaglomerular indices of 11, 10, and 7. Two of these 3 rats had a very mild hypertension. Renin responses of these 3 kidneys were +4 mm Hg, +7 mm Hg, and +7 mm Hg, respectively. The JG indices of the other 6 "untouched" kidneys were 4 or less, and the renin responses varied from −6 mm Hg to +1 mm Hg, indicating an almost complete absence of renin. The renin contents paralleled quite closely the juxtaglomerular indices. The JG index of normal kidneys from this strain averages 30 (S.E. of mean = ±1.9), indicating that the JG index of the "clipped" kidneys in this experiment is significantly elevated.

Exp. II. Kidneys of Wistar rats with desoxycorticosterone hypertension (Table II) were compared with kidneys of normal Wistar rats (Table III). It has been shown previously that juxtaglomerular granules virtually

TABLE II. Granulation of Juxtaglomerular Cells and Renin Content of the Kidney in 8 Rats with Desoxycorticosterone Hypertension.

	Final B.P. before obtaining kidneys (mm Hg)	JG index	B.P. response to kidney extract (mm Hg)
	154	2	−14
	150	1	− 4
	168	1	− 6
	160	2	− 6
	162	2	− 7
	154	1	−10
	162	1	− 5
	162	1	−14
Mean	159	1.4	− 8

TABLE III. Granulation of Juxtaglomerular Cells and Renin Content of 6 Normal Rat Kidneys.

	JG index	B.P. response to kidney extract (mm Hg)
	55	+52
	67	+52
	48	+22
	29	+18
	44	+20
	42	+21
Mean	48	+31

disappear in rats given desoxycorticosterone and a high sodium intake(3,4). In Table II, the JG indices and renin responses of 8 such hypertensive rats are presented. Average JG index in the group receiving desoxycorticosterone was 1.4 and no index was higher than 2 in any individual rat. Renin response averaged –8 mm Hg for the group. In this group, the extracts uniformly produced a drop in blood pressure of bioassay test animals, indicating an almost complete absence of renin in these kidneys. Virtual absence of juxtaglomerular granules corresponded quite well with the extremely low renin content.

In Table III, the results with the 6 normal control rats are given. The average JG index for the group was 48 and the average renin response was +31 mm Hg. There is an abundance of both renin and juxtaglomerular granulation in kidneys of these rats.

Discussion. Renin assays obtained on our rats receiving desoxycorticosterone confirm the observations of Gross and Sulser(7). However, renin assays on our rats with renal hypertension are not in agreement with those of Williams, *et al.*(8). In their studies the "ischemic" kidney of the rat usually did not have more extractable renin than the contralateral "untouched" kidney. Our experimental method was slightly different from theirs, which probably accounts for the difference in results. The fact that renin closely parallels granulation of juxtaglomerular cells, does not prove that these granules contain renin. The observation, however, is most compatible with such a hypothesis. It may be that both renin content and granules are separately and independently influenced by certain physiological states, such as a rise in the pressure of blood perfusing a kidney. Further experiments are necessary before the question can be finally resolved.

In kidneys with a very low juxtaglomerular index, the kidney extract usually produced a depressor response in the test animal. In these kidneys, the renin content is probably minimal, allowing depressor substances in the extract to become readily apparent. In these kidneys there is no doubt that depressor substances are present. It is possible that they are related to the depressor effects exerted by normal kidneys when introduced into the circulation of rats with renal hypertension(9). However, the presence of these depressor substances did not prevent hypertension in these rats.

Summary. Normal rat kidneys and kidneys with a narrowed renal artery have abundant granules in the juxtaglomerular cells and large quantities of extractable renin. On the other hand, kidneys of rats with desoxycorticosterone hypertension and the "untouched" kidney of rats with unilateral renal hypertension (resulting from narrowing of the contralateral renal artery) both have a virtual absence of juxtaglomerular granules as well as absence of extractable renin. There is so little renin in these kidneys that the renal extracts usually produce a depressor response. Thus, there exists a striking correlation between the amount of juxtaglomerular granulation and the amount of extractable renin in a kidney. This does not prove that these granules are composed of renin but the observations are compatible with such a hypothesis.

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Propagation of Mouse Hepatitis Virus (Gledhill) in Tissue Culture. (24536)

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The Gledhill strain of mouse hepatitis virus (MHV 1)(1,2) causes a mild, rarely fatal infection in weanling Swiss mice and a fatal infection in newborn mice. Host-specific viruses associated with hepatitis infections of duck(3-5), dog(6), and man(7) have also been described. However, only agents of canine hepatitis and of Buescher's mouse hepatitis(8) have been propagated *in vitro*(9-12). This communication describes procedures for propagation of MHV 1 in tissue cultures of mouse kidney explants. As MHV 1 did not cause consistent cytopathogenic effects in our tissue cultures, infectivity tests with newborn mice were used to detect the fate of this virus in serially passaged cultures.

Materials and methods. Stock virus. A 5 g pool of livers from suckling Swiss mice, which as newborns were infected 5 days previously *via* the peritoneal route, was homogenized in a Ten Broeck grinder with Hank's balanced salt solution (BSS) (10% w/v) containing streptomycin (0.2 mg/ml) and penicillin (200 units/ml). The chilled homogenate was centrifuged at 2,000 rpm for 30 minutes at 4°C. The partially clarified virus suspension was sealed in ampoules and stored in a dry-ice chest. *Virus titrations.* Serial decimal dilutions of stock virus were prepared in phosphate-buffered saline (PBS) (pH 7.2). Each dilution was inoculated (0.05 ml) *via* peritoneal route into a litter of 1-2 day old Swiss mice (5-8 mice/litter). Five days later surviving mice were sacrificed with ether. Liver damage was noted and the 50% infective dose (ID_{50})/0.05 ml of inoculum was calculated(13). Grossly affected livers were removed aseptically. They were frozen immediately and subsequently used for preparation of stock virus. Stock virus prepared and assayed by these methods had ID_{50} endpoints of $10^{-3.3}$ to $10^{-3.6}$. Attempts to achieve higher titres either by repeated freezing and thawing or by repeated extraction of liver tissues with PBS or BSS failed. *Tissue culture media.* The following media used for growth of kidney explant cultures and for virus propagation contained streptomycin (0.2 mg/ml) and penicillin (200 units/ml) and were adjusted to pH 7.6-7.8 with a 2.8% solution of sterile sodium bicarbonate: medium A, 5 parts mouse embryo extract (40% stock solution made in BSS), 10 parts LAH (5% stock solution lactalbumen hydrolysate), and 85 parts medium 199; medium B, 10 parts calf, beef, horse, or human serum, 10 parts LAH, and 80 parts medium 199. *Kidney explant cultures.* Kidneys of newborn Swiss mice were removed aseptically and washed twice in BSS, minced finely, rewashed, and suspended in BSS. The kidney mince was dispersed evenly over lower half of screw-cap test tubes (16 x 125 mm) containing a thin coating of chicken plasma and excess fluid was removed. Twenty-five minutes later 1 ml of medium A was added. The cultures were incubated at 37°C on roller drum apparatus (6-8 rph). Those cultures which showed proliferation within 18 hours and confluent sheets of cells within 120 hours were used for virus inoculation. Minced kidneys of 25 newborn mice yielded sufficient tissue for 16-25 explant cultures.

Results. Attempts to propagate MHV 1. Propagation of MHV 1 in the following established cell lines was unsuccessful: L strain of mouse fibroblast(14), human amnion(15), HeLa(16), and also commercially available trypsinized monkey kidney (Microbiological Asso.). Virus could not be detected either by cytopathogenic effects or by infectivity trials in newborn mice with cultures assayed from 1-22 days after inoculation. In most instances the virus disappeared within 12-24 hours and did not reappear after 2 or 3 serial passages. In control tubes with medium A

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TABLE I. Serial Passage of MHV 1 in Tissue Cultures of Mouse Kidney Explants.

Passage	Virus dilution	Growth of explants (hr)*	Theoretical† $-\log ID_{50}$ at 0 hr	Calculated $-\log ID_{50}$		
				0 hr	48 hr	96 hr
1	10^{1}	18	2.5	2.4	4.8	
2	10^{3}	18	2.8	3.0	4.6	
3	10^{4}	72	3.6	2.5		4.8
4	10^{5}	120	3.8	3.8		5.2
5	10^{7}	120	3.2	1.9		4.4
6	10^{8}	48	3.4	NT‡		4.7
7	10^{9}	48	3.7	3.7		4.1
8	10^{10}	72	3.1	3.5		4.8
9	10^{11}	72	3.8	3.6		4.2
10	10^{12}	48	3.2	2.7		4.3

* Mouse kidney explants were grown for indicated period in medium A (see text) prior to inoculation with stock virus (ID_{50} $10^{-3.5}$) and subsequently with successive passages.

† Theoretical ID_{50} endpoints were 0.1 of calculated titres of respective preceding cultures in all cases except passages 2 and 5 where titres were 0.01 of respective preceding cultures.

‡ Not tested.

and without tissue, the ID_{50} endpoint decreased from $10^{-2.5}$ to $10^{-0.6}$ after 6 hours. The presence of serums (medium B with horse or calf serum) delayed virus decay slightly. In other experiments, stock virus was inoculated into stationary flasks containing medium B with horse serum plus either minced kidneys or livers from newborn mice. Virus disappeared from liver cultures within 24-48 hours and from kidney cultures within 120 hours. Similar results were reported by Pollard(17) who demonstrated the effectiveness of minced kidney tissue for prolonged survival of PRI mouse hepatitis virus(18).

Serial passage of MHV 1 in mouse kidney explant cultures. These cultures were prepared with medium A for serial passage of MHV 1 (Table I). After 18-120 hours, nutrient fluids were removed from proliferating explant cultures. Cultures were inoculated with virus and reincubated. The original inoculum consisted of 0.1 ml of undiluted stock virus (ID_{50} $10^{-3.5}$). Following 30 minute adsorption period on roller drum, 0.9 ml of medium A was added to each tube. Duplicate tubes were removed at this time (0 hour) and 3-5 tubes were removed at intervals (48 or 96 hours) thereafter. The contents of each series were pooled, ground in Ten Broeck grinder, ampulated, and stored in a dry-ice chest prior to assay in newborn mice. Subsequently, 10 serial passages were made. For calculation of ID_{50} endpoints, the original inoculum of 10% liver homogenate (stock virus) was considered as undiluted. Except for serial passages 2 and 5, successive cultures were inoculated with 0.1 ml of their preceding cultures. The 2 exceptions were inoculated with 0.1 ml of 1:10 dilutions (made in PBS) of their respective preceding cultures. Theoretical ID_{50} endpoints at 0 hour are included in Table I for comparison with the 0 hour samples. For successive cultures, these values were 0.1 of the calculated ID_{50} endpoints of their preceding cultures in all cases except serial passages 2 and 5. For the latter cases, theoretical values were 0.01 of their respective preceding cultures. The ID_{50} endpoints of successively passaged cultures ranged from $10^{-4.1}$ to $10^{-5.2}$ with average of $10^{-4.6}$ (Table I). The theoretical average at 0 hour was $10^{-3.3}$ and varied slightly from the calculated 0 hour average $10^{-3.0}$. In several instances, theoretical titres were slightly higher or equal to the calculated 0 hour titres, possibly indicating decay or adsorption of virus by the proliferating explants. However, for passages 2 and 8, the reverse situation occurred and may be within the range of experimental error. Later experiments indicated that adsorption had occurred and that extracellular virus was infective for newborn mice.

Total time of virus growth (636 hours) during the 10 passages in tissue culture far exceeded virus decay rate at 37°C in medium A and indicated that virus propagation had occurred. In addition, virus inoculum was diluted to 10^{-12} of its original concentration.

TABLE II. Comparison of Infectivity of Successive Culture Fluids with Whole Kidney Explant Cultures.

Series	Culture fluids		Whole cultures
	Hr	$-\log ID_{50}$	$-\log ID_{50}$
			Hr
a	0	2.6*	2.7 (0)
	120	2.8	
b	72	4.5	4.5 (72)
	"	3.8	
	96	3.3	
c	96	4.3	4.3 (96)
	72	3.3	
	"	3.9	

* Theoretical ID_{50} of intracellular virus which remained after removal of 0 hr culture fluids was $10^{-0.6}$.

Cytopathogenicity was noted in several instances but such effects occurred erratically. Occasionally, cells became enlarged, refractile, and grape-like clusters were formed as has been described for canine infectious hepatitis(9). Cell destruction usually occurred around the periphery of explant growth. These observations are being studied in greater detail.

Infectivity of tissue culture fluids. Kidney explants were grown in medium A for 48 hours at which time confluent sheets of cells had formed. Media were removed and explant cultures were inoculated with 0.1 ml of serial passaged tissue culture 9 (ID_{50} $10^{-4.2}$). After 30 minute adsorption period on roller drum. 0.9 ml of medium A was added to each tube (theoretical ID_{50} $10^{-3.2}$). Nutrient fluids were removed for assay and replaced with 1 ml of medium A at time intervals (Table II). Three series of cultures (a, b, and c) were prepared. It should be noted that 0 hour cultures (series a) were rinsed twice with 1 ml of PBS following adsorption period. For comparison with the titres of culture fluids, whole cultures were assayed after 0, 72, and 96 hours.

The data shown in Table II suggest that 1) virus is adsorbed by proliferating explant cultures, 2) virus propagation had occurred, and 3) only extracellular virus may be infective. Adsorption of virus is shown by decrease in titre at 0 hour (series a) from a theoretical ID_{50} endpoint $10^{-3.2}$ to $10^{-2.6}$. The difference ($10^{-0.6}$) represents the potential ID_{50} adsorbed by kidney explants and which remained after rinsing the inoculated explants with PBS. Further evidence that adsorption and propagation had occurred is shown by subsequent rise in titre (ID_{50} $10^{-2.8}$) after 120 hours. In series b and c, the final dilution of the original inoculum after 3 successive changes of nutrient medium exceeded the ID_{50} endpoint of the inoculum and total time of these experiments (240 hours) exceeded decay time of the virus. The similarity of titres obtained with entire cultures and with culture fluids alone at comparable time intervals (0, 72, and 96 hours) suggests that only the culture fluids contain the mature virus.

Summary. The Gledhill strain of mouse hepatitis virus (MHV 1) was passaged through 10 successive tissue cultures of newborn mouse kidney explants. As MHV 1 did not cause reproducible cytopathogenic effects, infectivity tests with newborn mice were used to detect propagation of this virus in serially passaged cultures. Observations suggest that virus was adsorbed by the proliferating explants and that cell-free culture fluids were infective. Total time of virus growth far exceeded decay rate of the virus incubated without tissue. After the tenth passage, the virus had been diluted 10^{12}-fold which exceeded the extinction point of the original inoculum.

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Formation of a Pharmacologically Active Substance from Plasma Protein.* (24537)

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A number of investigators(1,2) have shown that vasodilator polypeptide, bradykinin, can be produced by incubating plasma protein with crystalline trypsin. Also that an enzyme capable of producing bradykinin upon incubation with plasma protein can be released from salivary gland cells by stimulation of the chorda tympani(3) and that bradykinin so produced has a role in regulation of blood flow in the submandibular salivary gland. It occurred to us that the enzyme α-amylase might be important in production of bradykinin, since salivary glands are rich in α-amylase. Furthermore, fraction IV-4 of plasma protein which contains bradykininogen, the precursor of bradykinin, is rich in glycoprotein which could well be susceptible to hydrolysis by α-amylase. Therefore it seemed conceivable that the action of α-amylase on glycoprotein might lead to production or activation of bradykinin. Saliva incubated with blood produced vasodilator principle bradykinin(3) and α-amylase is present in saliva along with small amounts of other enzymes.

Materials and methods. Fraction IV-4 of plasma protein was obtained through joint cooperation of American Red Cross and E. R. Squibb. Enzymes and enzyme inhibitors were obtained from commercial sources except salivary amylase, which was prepared from pooled saliva and partially purified by the method of Bernfeld(4). Detection and extent of formation of the pharmacologically active material in the incubation mixture was measured by ability to contract isolated segments of guinea-pig ileum suspended in 10 ml organ bath and attached to frontal writing lever for recording on smoked drum of kymograph. The bath fluid was Tyrodes' solution at 37°C. The effect of pharmacologically active polypeptides on blood pressure was studied in dogs and rabbits anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and urethane (1-1.5 g/kg), respectively. Pressure was recorded from cannulated carotid artery with a mercury manometer. All injections were made *via* cannulated femoral vein. Formation of pharmacologically active material was carried out by incubating equal volumes of 0.5% α-amylase and 0.5% fraction IV-4, both dissolved in Tyrodes solution at 37°C. At appropriate times aliquots were removed and tested for activity on the guinea pig ileum. The active material was in contact with the tissue for 60 seconds. Aliquots of the incubation mixture, removed at appropriate times, were also injected I.V. into anesthetized dogs and rabbits for measurement of effects on blood pressure. Formation of

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bradykinin was accomplished by incubating equal volumes of 0.5% fraction IV-4 and 0.03% crystalline trypsin both dissolved in Tyrodes' solution at 37°C. When enzyme inhibitors were used they were preincubated with appropriate enzymes for 15 minutes before mixing with the plasma protein fraction. Enzyme inhibitors were used in concentration equal to that of the enzyme.

Results. A pharmacologically active substance was formed by incubating α-amylase (NBC #9342) with fraction IV-4 of plasma protein. Maximum production of the active material was attained after approximately 20 minutes' incubation. This is contrasted with formation of bradykinin by action of crystalline trypsin on fraction IV-4 of plasma protein in which time of maximum formation was between 2 and 5 minutes incubation. Rate of destruction of the active substance produced by incubating α-amylase with fraction IV-4 of plasma protein, by enzymes present in the incubation mixture, proceeded much more slowly than its formation, requiring approximately 100 minutes for the activity to become negligible. This was contrasted with rate of destruction of bradykinin in crystalline trypsin:fraction IV-4 incubation mixture in which it was found that bradykinin activity was negligible after 20 minutes incubation. Prolonged incubation of enzymes or substrate alone did not produce any active product. All Cohn fractions of plasma protein except fraction IV-4 were inactive as substrates for enzymatic formation of the pharmacologically active substance.

If a specific α-amylase inhibitor‡ was added to the fraction IV-4:α-amylase incubation tube, in concentration equal to that of the α-amylase present, formation of the active substance was completely inhibited. Also, trypsin inhibitor‡ prevented formation of bradykinin when added in concentration equal to that of the crystalline trypsin. In addition, α-amylase inhibitor had no effect on formation of bradykinin and trypsin inhibitor had no effect on formation of the active substance obtained by incubating α-amylase with fraction IV-4 of plasma protein. It would therefore seem that the pharmacologically active substance was different from bradykinin. This possibility was confirmed by experiments testing the activity of these substances on dog and rabbit blood pressure. Incubation mixtures of fraction IV-4 and crystalline trypsin when injected into dogs and rabbits, gave the hypotensive response typical of bradykinin. When incubation mixtures of fraction IV-4 and α-amylase were injected into dogs and rabbits a pressor response was obtained. Since this active substance differs from bradykinin we have provisionally named it Substance A.

‡ Obtained from Worthington Biochemicals Corp., Freehold, N. J. The trypsin inhibitor was a 5-times crystallized soy bean trypsin inhibitor.

TABLE I. Comparative Ability of Different Alpha Amylase Preparations to Form a Pharmacologically Active Material when Incubated with Fraction IV-4.

Source of alpha amylase	Time of max formation (min.)	Relative activity*
Nutritional Biochemicals Corp.		
#9342	18	100
#4416	10	43
#4261	6	169
Southeastern Biochemicals	12	122
Mann #A-5074	25	93
K and K Labs. #9307F	5	67
Salivary (partially purified)	10	60

* Calculated using Nutritional Biochemicals #9342 as standard alpha amylase.

Table I illustrates ability of different α-amylase preparations from various sources to form a pharmacologically active material when incubated with fraction IV-4. All of the α-amylase preparations tested were able to produce an active substance. β-amylase was found to be totally inactive under the conditions of our test.

Summary. Evidence has been presented on formation of a pharmacologically active substance from fraction IV-4 of plasma protein by the action of an α-amylase preparation. Formation of the active substance stimulates the isolated guinea pig ileum and produces a pressor response on dog and rabbit blood pressure. It differs from bradykinin since that substance is a vasodilator. The active substance produced by action of α-amylase on fraction IV-4 of plasma protein has been pro-

visionally designated as Substance A. Further studies concerning enzymatic formation as well as purification and characterization of Substance A will be published.

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Serial Cell-Free Passage of a Radiation-Activated Mouse Leukemia Agent.* (24538)

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Mice of the C3H inbred line do not usually develop "spontaneous" leukemia. Although the incidence varies in different laboratories, in our colony of C3H or C3H(f) mice (both of Bittner substrain) the incidence during past 10 years has not exceeded 0.5%(1). However, fractionated total body irradiation (150 r, 4 to 5 times, at weekly intervals) resulted in development of leukemia in over 50% of C3H mice after a latency period of approximately 6 to 7 months. Leukemia thus induced could then be transmitted, by filtrates, into newborn C3H mice; incidence was significant (11%), but not too high, and many extracts were inactive on inoculation tests (2). In our previous studies, some filtrates prepared from spontaneous Ak or C58 leukemias were also inactive on inoculation tests; however, selecting a potent filtrate and passing it serially through newborn mice, resulted eventually in development of a highly potent "passage A" leukemic virus inducing up to 90% leukemia after a latency of 3 to 4 months following inoculation into newborn or suckling C3H mice(3,1). Faced now with an apparently similar situation, we selected one of the more potent extracts among those prepared from radiation-induced C3H leukemias, and passed it serially through newborn C3H mice.

Materials and methods. All mice used were C3H or foster nursed C3H(f) mice, both of the Bittner subline. *Origin of leukemic virus strain.* The donor serving for preparation of the initial filtrate was a C3H female which, at age of 1½ months received a series of total body x-ray irradiation, 150 r each at weekly intervals, for 4 consecutive weeks. Five months after last irradiation, this mouse developed a very large thymic lymphosarcoma, and was then used as donor for preparation of the initial filtrate. *Preparation of filtrates for inoculation.* The leukemic donors were sacrificed by ether inhalation, and, without delay, parts of thymic and mesenteric tumors, peripheral lymph nodes, livers and spleens, were removed aseptically, weighed, and ground by hand in mortar with chilled, sterile physiological saline added, to obtain cell suspensions of 20% concentration. After centrifugation at 3,000 rpm (1,400 x g) for 15 minutes, the supernate was removed and again centrifuged at 9,500 rpm (7,000 x g) for 5 minutes. The second supernate (10 to 12 ml) was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli*, and passed through Selas, porosity 02, porcelain filter candles, under vacuum pressure of approximately 20 mm mercury. All resulting filtrates were bacteriologically sterile, as evidenced by inoculating tryptose phosphate broth incubated for 24 hours; it was reasonable to assume, therefore, that no cells passed through filter candles. Kept at 0°C, most extracts

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were used within a few hours, none later than after 24 hours. *Inoculation of filtrates.* Newborn, less than 16 hours old, or suckling 1 to 5 days old C3H, or C3H(f) mice, were inoculated subcutaneously or intraperintoneally. The sexes were separated at weaning time. Mice that died when less than 6 weeks old, were not included in the tabulation.

Results. Serial cell-free passage of leukemic agent from host to host. Only a cell-free passage, by means of filtrate, consisting of successful transmission of leukemic agent from host to host, was considered a consecutive "passage" and given a passage number. As in previous studies, this procedure was adopted, because it appeared questionable whether cell-transfer would either increase, or only sustain the infective potency of the leukemic agent. The passage agent was designated by the letter "X".

The *first passage* filtrate, prepared from a C3H female in which leukemia was induced by total body x-ray irradiation, was inoculated intraperitoneally into 2 C3H litters consisting of 8 mice less than 15 hours old. As a result 3 mice developed generalized lymphatic leukemia at 6½, 10½ and 11½ months respectively. The remaining mice died without signs of leukemia or tumors at 14½ months of age.

Second passage. A filtrate was prepared from one of the leukemic donors from the preceding passage, and inoculated subcutaneously into a litter of C3H mice consisting of 7 mice less than 16 hours old. Six mice developed generalized lymphatic leukemia at ages varying from 5 to 13 months.

Third passage. Two filtrates were prepared from 2 leukemic donors from the preceding passage, and inoculated into 4 litters varying in age from 2 hours to 2½ days. Of 16 mice, 13 thus far, (81%) developed generalized leukemia when 4 to 8 months old, and 3 are still alive and well at 8 months of age.

Fourth passage. Two filtrates were prepared from leukemic donors of the preceding passage, and inoculated into 9 mice varying in ages from 4 hours to 3 days. Four of these, thus far, developed generalized leukemia at 3 and 4 months respectively. The remaining 5 are still well at 4½ months of age.

Morphology of passage X filtrate induced leukemia. Mice which developed leukemia as a result of inoculation of passage X filtrates presented typical picture of generalized lymphatic leukemia, with enlargement of peripheral lymph nodes, large thymic and often also mesenteric tumors, enlarged spleens and livers. Most livers examined microscopically, showed typical lymphocytic infiltration around the large vessels. Peripheral (tail) blood counts were made on 11 leukemic mice. The number of white cells varied from 5,850 to 44,750/mm^3 (average 21,636 as compared with 10,355 in normal C3H mouse). Almost all leukemic blood smears showed presence of lymphoblasts (2%), and smudge cells, and most of them also nucleated red cells in peripheral blood; the dominant white cell was the lymphocyte, many of them showing atypical forms. All leukemic mice showed moderate to marked anemia (average 9.6 g Hb/100 ml as compared with 14.7 in the normal C3H mouse).

Controls. In a control group, 186 newborn C3H mice were inoculated with normal C3H organ extracts, and only 1 (0.5%) developed leukemia at 17 months of age, but 14 (7.5%) developed parotid gland tumors at 4.5 months average age(2).

One or two leukemic viruses? We were confronted, therefore, with the fact that in C3H mice, leukemia could be induced by a) inoculation of passage A(3) leukemic virus which originated from spontaneous Ak leukemia and was then passed serially through newborn C3H mice, or b) inoculation of a filtrate designated "passage X," which originated from radiation induced C3H leukemia. Incidence of induced leukemia was higher and the latency period shorter, when passage A filtrates were inoculated. Since, however, morphological differences between these 2 groups of leukemia were not sufficiently consistent to permit a basis for distinction, the question remained open whether we were not faced with the same disease, induced by the same virus, harvested from different sources. As a working hypothesis it was possible to assume that we were dealing with 2 distinct viruses; such

TABLE I. Results of Neutralization* *In Vitro* of Passage A Leukemic Virus with Inactivated (56°C ½ Hr) Guinea Pig and Rabbit Immune and Normal Serum.

	Pass. A leuk. immune serum		X-ray induced leuk. immune serum		Normal serum		Controls, leuk. fil.†	
	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %
Rabbit serum	25	8	26	23	14	71	32	74
Guinea pig serum	44	34	47	85	17	41	54	78

* 20% passage A leukemic filtrate mixed 1:1 with undiluted serum, incubated at room temp. (22°C) for 30 to 60 min., then at 0° from 2 to 20 hr. All inoculations subcut. (138) or intraper. (121).

† Mixed 1:1 with physiol. saline.

All inoculated mice were of C3H or C3H(f) line (Bittner substrain); avg age at inoculation, 3 days. 22 mice died without signs of leukemia at avg age 9 mo. 93 mice surviving and well at present, avg age 11 mo.

Avg age leukemia developed: 4 mo in the controls and normal serum group; 4.7 mo in both immune serum groups.

an assumption, however, remained to be proven.

Attempt to neutralize mouse leukemia virus by a specific serum. A group of young, adult rabbits and guinea pigs, received at 7 to 10 days intervals, 6 to 8 intraperitoneal injections of leukemic filtrates prepared from passage A leukemic C3H donors. Another group of rabbits and guinea pigs received simultaneously a similar number of intraperitoneal injections of filtrates prepared from passage X leukemia, or of filtrates prepared from C3H donors in which leukemia was induced by total body x-ray irradiation. Both groups of rabbits and guinea pigs were bled 7 to 10 days after last injection. As a control, normal serum was obtained from untreated rabbits and guinea pigs. Serum from rabbits, or guinea pigs, in each group, was pooled, and used either fresh, or after inactivation at 56°C for 30 minutes. The undiluted serum from each group was then mixed 1:1 with a freshly prepared 20% passage A leukemic filtrate, incubated at room temperature (22°C) for 30 to 60 minutes, then for additional 2 to 20 hours at 0°C, and inoculated into suckling, 1 to 7 day old, C3H mice.

Fresh immune guinea pig passage A, or passage X, serum neutralized the passage A leukemic agent. Of 36 mice inoculated with leukemic filtrate mixed with fresh passage A serum, none developed leukemia; of 33 mice inoculated with leukemic filtrate mixed with fresh passage X serum, only 7 developed leukemia at 5 months of age. This neutralizing action of both immune sera was not specific however, since normal, fresh guinea pig serum had a similar neutralizing effect: thus, of 17 mice inoculated with leukemic filtrate mixed with normal guinea pig serum, one developed leukemia; (all 78 surviving mice are now in good health at 7.5 months of age). In a simultaneous control group, 18 mice were inoculated with leukemic filtrate mixed 1:1 with physiological saline solution, and all developed leukemia at average age of 3 months.

The results were different, when inactivated (56°C for 30 minutes) serum was used for neutralization tests (Table I). The passage A immune rabbit serum had a marked neutralizing effect on passage A leukemic agent, only 8% of inoculated mice developing leukemia, as compared with 74% in the control group, and 71% or 23% respectively in groups where normal or passage X rabbit sera were used.

Discussion. Experiments here reported suggest that normal, healthy mice of the low-leukemic C3H line, may carry a masked, usually non-pathogenic, leukemic agent. Triggered by ionizing radiation, this agent may become pathogenic, causing leukemia in its carrier host. Such an agent may then be transmitted, by filtrates, to other C3H mice, provided that it is inoculated into newborn hosts. The potency of filtrates prepared from different C3H donors with radiation-induced leukemia may vary considerably. Of 18 filtrates prepared from individual C3H donors with radiation induced leukemia, and inoculated into newborn C3H mice, 11 proved to be active on inoculation tests(2). It was ap-

parent therefore that a transmissible, filterable leukemogenic agent could be activated in at least 11 different C3H mice by total body x-ray irradiation. Selecting a potent extract, the agent could be passed serially through 4 consecutive cell-free passages of newborn C3H hosts.

Whether the filterable leukemic agent, designated "passage X" is distinct from leukemic virus designated passage A which originated from spontaneous Ak leukemia and which has been also passed serially in C3H mice, remains to be determined. Since the concentration of the virus in extracts used is not known, it is not possible to differentiate virus A from virus X on the basis of serum neutralization tests here reported. Thus, the assumption that the leukemic viruses A and X are distinct, although possibly related, remains a working hypothesis, and still requires experimental confirmation.

Summary. 1. A filterable leukemic agent designated "virus X" recovered from a C3H female in which leukemia was induced by x-ray irradiation, was passed serially through 4 consecutive cell-free inoculations of suckling C3H mice. 2. Of 40 mice inoculated, 26 thus far developed leukemia (65%) at ages varying from 3 to 11 months. 3. Inactivated (56°C ½ hr) rabbit and guinea pig serum, prepared with agent X filtrates, only partially neutralized passage A leukemic agent; passage A immune rabbit serum had a distinct, though not complete neutralization effect on the passage A agent.

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Site of Reaction of Wax Bean Hemagglutinin with Rabbit Erythrocytes.* (24539)

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Many plant seeds contain substances which agglutinate animal erythrocytes(1). Experiments reported here reveal several features of rabbit erythrocyte agglutination common to wax bean hemagglutinin (WBH) and to influenza and Newcastle Disease viruses. This suggests that both may act on the same site of the red cell membrane.

Materials and methods. Preparation of WBH. A partially purified preparation was obtained as follows. To 50 g of finely pulverized stringless wax beans‡ (*Phaseolus vulgaris*) was added 500 ml distilled water, and pH of suspension was adqusted to pH 7. Insoluble material was removed from suspension by centrifugation, and pH of supernatant was acidified to pH 4.6. Material precipitating out at this pH was discarded, and the supernatant fully saturated with $(NH_4)_2SO_4$. The precipitated protein was dialyzed against distilled water and finally lyophilized. The *receptor destroying enzyme* (RDE) was prepared from *Clostridium perfringens* as described by Popenoe and Drew(2). *Virus-treated blood cells* were prepared by adding 10 ml of chorioallantoic fluid [obtained from 10-day-old embryonated eggs inoculated with influenza (PR 8) or Newcastle Disease (NDV) virus§ and harvested after incubation at 37° for 48 hours] to 30 ml of 1.5% suspension of trypsinated rabbit erythrocytes(3). 0.9% NaCl replaced

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‡ Purchased from Farmer Seed and Nursery Co., Faribault, Minn.

§ Generously provided by Dr. J. T. Syverton, Dept. of Bacteriology, Univ. of Minnesota, Minneapolis.

TABLE I. Substances Showing Inhibition of Agglutination by WBH.

Substance tested	Inhibitory activity, I.U./mg	Sialic acid, %	Source or method of preparation
Ovomucin	520	5.8	(5)
Ovomucoid	10	2.0	(6)‡
Orosomucoid	350	16.1	(7)§
Serum viral inhibitor	475	12.2	§
Rabbit serum	16*	16.0*	
Bovine "	100*	26.0*	
Meconium	300	4.5	(8)‖
Sialyl-lactose	170	47.5	(9)
Sialic acid (meconium)	0	100 †	(10)
(ovomucin)	2	85	(11)

* Inhibitor activity and sialic acid expressed as I.U./ml and mg % respectively.

† Assumed to be 100%; used as standard in determination of sialic acid(4).

‡ Purchased from Worthington Biochemical Corp., Freehold, N. J.

§ Gift from Dr. R. J. Winzler, Univ. of Illinois, Chicago.

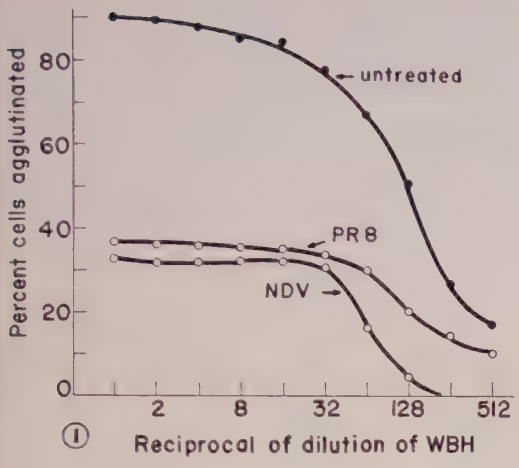
‖ Gift from Dr. R. K. Silver, Univ. of Pennsylvania, Philadelphia.

the virus for control. After 2 hours at 4°, the cells were washed twice with cold 0.9% NaCl and resuspended in 50 ml of saline. The cells were held overnight at 37°, washed 3 times, and finally suspended in sufficient 0.9% NaCl to give a red cell concentration of 1.5%. *RDE-treated serum* was prepared by mixing 1 ml rabbit serum, 1 ml RDE solution (replaced by 0.015M $CaCl_2$ in control), and 3 ml 0.015 M $CaCl_2$. Following incubation for 2 hrs at 37°, enzymic action was stopped by heating at 56° for 90 min. Filtrates, obtained by adding 5 ml of 5% trichloroacetic acid to 2 ml aliquots of each solution, were analyzed for sialic acid, the difference between RDE-treated and control sera representing net enzymic release of sialic acid from the serum. The remainder of each solution, after dialyzing against 0.9% NaCl, was tested for inhibitor activity. *Hemagglutination inhibition.* The substance to be tested was dissolved in 0.9% NaCl, and 2-fold serial dilutions of this solution were made up in final volume of 0.5 ml using 0.9% NaCl as the diluent. To each tube was added 0.5 ml of WBH solution containing 2 γ protein equivalent to 25 hemagglutinating units as defined by Liener(3). After 30 min. at room temperature, 1 ml of 1.25% suspension of trypsinated rabbit erythrocytes was added to each tube and mixed. The tubes were read photometrically at the end of 2-2½ hrs., and dilution of inhibitor corresponding to 50% sedimentation of cells was calculated(3). One inhibitor unit (I.U.) was arbitrarily defined as that amount of test substance which caused 50% inhibition of complete agglutination under the conditions specified above. The source or method of preparing the various substances tested for inhibitory activity against WBH is shown in Table I. *Sialic acid* content of these materials was determined according to the method of Svennerholm(4).

Results. Inhibition by mucoproteins. In Table I is presented a partial list of substances tested for inhibitory effect on hemagglutinating activity of WBH.‖ In general the most potent inhibitors of WBH belonged to that class of soluble mucoproteins which contain sialic acid and act as inhibitors of viral agglutination(12,13). Inhibition by sialyl-lactose may be compared to inhibition of viral agglutination by a similar, if not identical, compound isolated by Kuhn and Brossmer (14) from cow colostrum. Sialic acid itself, which did not inhibit WBH, likewise has no inhibitory effect on viral agglutination(15). These features of similarity between WBH and viral agglutination suggest a mechanism of inhibition analogous to that postulated for the virus, namely that mucoproteins, by virtue of their chemical constitution (involving sialic acid), combine with virus (or WBH) and prevent their attachment to similar sites on the surface of the red cell.

Agglutination of virus-treated cells. Erythrocytes agglutinated by influenza virus, and from which the latter has been spontaneously eluted, can no longer be agglutinated by the same virus(16). This has led to the concept that virus enzymically "destroys" the receptor site on the cell membrane involved in agglutination. If this site is identical with that involved in the combination of WBH with red cells, pretreatment of the latter with virus should reduce their susceptibility to agglutina-

‖ Not included in Table I are about 20 simple sugars and oligosaccharides which displayed little or no inhibition. Of these only α-lactose and D-galactose were found to exhibit measurable inhibition (0.2 and 0.3 I.U./mg respectively).



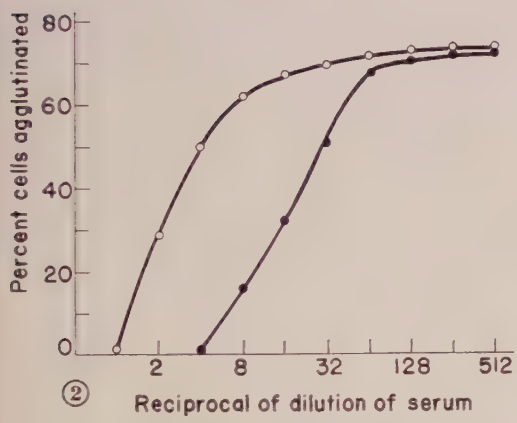


FIG. 1. WBH agglutination of rabbit erythrocytes pretreated with influenza (PR8) or Newcastle disease (NDV) compared with untreated cells. Two-fold serial dilutions of WBH were made from stock solution containing 20 γ protein/ml.

FIG. 2. Inhibition of WBH agglutination by RDE-treated rabbit serum (○—○) compared to untreated serum (●—●). Two-fold serial dilutions of sera were made from final solutions equivalent to 0.2 ml serum/ml. Level of WBH in each tube = 2 γ protein.

tion by WBH. The results of an experiment designed to test this hypothesis (Fig. 1) showed that untreated cells were more readily agglutinated by WBH than cells which had been pretreated with PR 8 or NDV virus. Concentrations of WBH which caused almost complete agglutination of untreated cells caused only partial agglutination of virus-treated cells. Attempts to demonstrate a similar effect by replacing virus with RDE were not successful. It had been previously noted that sensitivity of red cells to agglutination by WBH is considerably enhanced by treatment with proteolytic enzymes. Since no claim can be made for the purity of the RDE preparation used here(2), the possibility exists that blood cells may have been sensitized by peptidases contaminating this preparation of RDE(17), thus obscuring any effect RDE might have had in destroying the receptor site.

Inhibition by RDE-treated serum. Mucoprotein viral inhibitors, subjected to the action of RDE, no longer inhibit viral agglutination, an effect accompanied by release of sialic acid(12,13). Fig. 2 presents the results of an experiment in which rabbit serum, an effective inhibitor of WBH (Table I), was treated with RDE and tested for residual inhibition. A distinct difference in inhibition was obtained between the RDE-treated serum and the untreated control. Arbitrarily selecting 50% agglutination as a reference point, the dilutions corresponding thereto for the treated and untreated sera were 1:4 and 1:32 respectively. From sialic acid determinations, it was calculated that 73% of the protein-bound sialic acid residues originally present in the serum had been cleaved by RDE.

Discussion. Gottschalk(12) summarized the salient features of virus-cell-mucoprotein interaction as follows: (a) certain mucoproteins inhibit viral agglutination of red cells, (b) when pretreated with virus or purified enzyme (RDE), mucoproteins no longer inhibit and cells are no longer agglutinated, and (c) concomitant with these effects, a small molecular weight compound (sialic acid) is released from mucoprotein or cellular stroma.

Evidence has been presented here to sustain the view that the phenomenon of phytoagglutination (at least in the case of WBH) conforms rather strikingly to these characteristics. Thus, (a) all mucoproteins which inhibited WBH agglutination have been reported to be potent inhibitors of viral agglutination, (b) blood cells treated with 2 different types of virus became more refractory to agglutination, and finally (c) inhibitory activity of serum against WBH was considerably reduced (8-fold) when pretreated with RDE, an effect which was associated with release of about

75% of the bound sialic acid of serum. By analogy, therefore, it may be presumed that the combination of WBH with red blood cells involves the same or similar site on the surface of the cell which serves as a substrate for the viral enzyme or RDE.

There are two further observations of a negative nature. First, WBH could not be eluted from the erythrocytes once agglutination had occurred, and secondly, WBH was unable to cleave sialic acid from the various mucoproteins shown in Table I or from sialyllactose. Of the 3 successive steps involved in viral agglutination-adsorption leading to agglutination, enzymic destruction of receptor site, and elution of virus—the enzymic and elution stages can therefore be clearly excluded in the case of WBH. Thus the mode of action of WBH most closely resembles that of the heat-inactivated or "indicator" virus which retains the capacity to agglutinate red cells, an effect which can be inhibited by mucoproteins, but which can no longer be eluted due to the loss of its enzymic function.

Summary. Mucoproteins known to be inhibitors of viral agglutination inhibited agglutination of rabbit erythrocytes by the wax bean hemagglutinin (WBH). Pretreatment of red blood cells with influenza (PR 8) or Newcastle Disease virus reduced susceptibility of such cells to agglutination. The enzymatic release of sialic acid from rabbit serum by a receptor-destroying enzyme prepared from *Cl. perfringens* was accompanied by a loss in inhibitory activity of serum against WBH agglutination. These and related observations suggest that the site of reaction of WBH with erythrocytes is similar, if not identical, to that of the viral receptor site on the surface of the cell.

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A Mechanism of Action for Antithyroid Activity of Reserpine.* (24540)

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Since the introduction of reserpine as a therapeutic agent, reports have appeared ascribing an antithyroid activity to this substance. Chronic reserpine therapy reportedly resulted in "normalization" of the basal metabolic rate in 15 hyperthyroid patients(1), and in peripheral inhibition of thyroxin by reserpine(2,3), as well as a more direct action by reserpine upon the thyroid(4,5). Determination of basal metabolic rate or degree of iodine

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TABLE I. Comparative Drug Effects upon Dehydrogenase Enzyme Activity of Thyroid.

Treatment	No. of animals	Avg wt of dried thyroids, mg	μg of dye reduced/mg dried tissue, mean ± S.E.	P
(a) Normal controls	28	2.1	14.2 ± .6	
(b) Reserpine	30	2.5	10.9 ± .5	*vs* (a) <.0001
(c) Synthroid Na	15	2.5	7.9 ± .7	*Idem*
(d) Thiouracil	15	5.4	30.9 ± .7	"
(e) Thyrotropar	14	2.0	20.3 ± 1.2	"
(f) Reserpine + thiouracil	10	4.1	29.6 ± 2.1	*vs* (b) $<2 \times 10^{-12}$
(g) " + Thyrotropar	20	1.6	19.1 ± .8	*Idem*

uptake was employed in the above reports for measuring any effect by reserpine upon the thyroid gland. In the present investigation the effect of reserpine upon endogenous dehydrogenase enzyme activity of the thyroid was quantitated. These results were compared to results obtained with substances whose mechanism of action upon the thyroid is known.

Methods. Male and female adult rats of our inbred colony of the Denver Strain were used. No sex difference was found in control or treated animals. Endogenous dehydrogenase enzyme activity was quantitated employing the tetrazolium technic(6) as modified for tissue slices(7). Triphenyltetrazolium chloride was used as indicator in a modified Kreb's solution(8). Incubation was carried out aerobically with shaking at 37°C for 2 hours. After killing the animal by a sharp blow on back of the head, both lobes of the thyroid were immediately dissected free and incubated without further sectioning. The amount of dye reduced by both lobes was determined colorimetrically after extraction with acetone. This value was converted to μg of dye reduced/mg of air dried tissue. Pure powdered reserpine‡ was dissolved in a minimum of glacial acetic acid and brought to the desired volume with 10% propylene glycol. The previously reported dosage schedule of 100 μg/kg/day administered subcutaneously for 14 days was employed(9). Thiouracil was given in the drinking water as a 0.1% solution for 10 days(10). Thyrotropin, as Armour's Thyrotropar, was administered intramuscularly at a dose of 2 units/rat in 4 divided doses over a 24 hour period(11). Synthroid Na, the synthetic l-thyroxin preparation of Travenol Labs, was suspended in normal saline and injected subcutaneously daily for 8 days at 50-75 μg/rat/day. To observe effects of combined reserpine-thiouracil administration, thiouracil was added to drinking water 4 days after initiation of daily reserpine treatment, and both substances given together for another 10 days. Thyrotropin, when administered together with reserpine, was given on the last day of the regular 14-day reserpine schedule.

Results. Mean values of enzyme activity obtained with each experiment are given in Table I. P values were calculated according to conventional methods(12).

Reserpine with the dosage and time schedule employed significantly lowered endogenous dehydrogenase enzyme activity of the thyroid. It has been reported that prolonged administration of reserpine in rats produces slight thyroid enlargement, indicating histologically mild functional inhibition(13). Under our conditions, no histological or macroscopic changes from the normal were seen.

Administration of synthetic l-thyroxin also reduced enzyme activity. Histological examination revealed a mildly resting gland. Thiouracil and thyrotropin significantly raised enzyme activity. With thiouracil administration the gland was typically enlarged and highly vascular. Histological examination of these glands revealed active, hyperplastic thyroid tissue. Thyrotropin, as administered, produced no glandular enlargement or hyperplasia. Microscopically an active epithelial structure was present. Concomitant adminis-

‡ Powdered reserpine (Serpasil) was obtained through courtesy of Dr. F. F. Yonkman, Ciba Pharmaceutical Products.

tration of reserpine with either thiouracil or thyrotropin did not alter the effects of these substances upon either enzyme activity or histological findings.

Reduction by exogenous thyroxin of thyroid dehydrogenase enzyme activity was probably due to decreased output of thyrotropin by the pituitary, since administration of exogenous thyrotropin and stimulation of endogenous thyrotropin secretion by thiouracil administration caused an increase in enzyme activity. It thus appears that dehydrogenase enzyme activity of the thyroid reflects degree of thyrotropin activity upon the thyroid.

The reduced enzyme activity, with reserpine administration, does not appear to be due to a direct action upon the thyroid. Administration of thyrotropin, following 13 days of reserpine injection, resulted in a significant increase in enzyme activity over that observed with reserpine alone. In addition, reserpine did not reduce the high levels of enzyme activity due to thiouracil administration. These observations are interpreted as showing that reserpine does not block the action of thyrotropin within the thyroid. It would appear that dehydrogenase enzyme activity of the thyroid is related to the action of thyrotropin on cellular elements of the gland, without regard to thyroxin production by the gland. The high degree of cellular hyperplasia and known reduction of thyroxin secretion in thiouracil treated animals substantiate this belief.

Our data indicate that reserpine has a significant effect upon endogenous dehydrogenase enzyme activity of the thyroid. Due to reserpine's known effects upon higher centers in the central nervous system(13), and the presently suggested relation to thyrotropin activity, it is proposed that the mechanism of action for this reduction by reserpine is *via* the pituitary, to produce a decreased thyrotropin elaboration or release.

This effect of reserpine upon the thyroid *via* the pituitary, although significant, does not appear to be powerful in nature. It did not inhibit the rise of enzyme activity when given with thiouracil. However, some consideration should be given to it when long term reserpine therapy is contemplated. This might be especially true in view of the report that chronic reserpine administration in euthyroid guinea pigs reduced the basal metabolic rate to that of hypothyroid levels(3).

Summary. 1) Endogenous dehydrogenase enzyme activity of the thyroid was quantitated employing the tetrazolium technic. Reserpine and thyroxin significantly lowered enzyme activity, whereas thyrotropin and thiouracil significantly raised it. Thyrotropin, either exogenous or endogenous in origin, prevented reduction of enzyme activity achieved by administration of reserpine alone. 2) The suggestion that these effects are a reflection of thyrotropin activity upon the thyroid is discussed. It is proposed that reserpine does have an antithyroid action and that this action is the result of its effect upon the elaboration or release of thyrotropin by the pituitary.

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Relationship of Vitamin B_6 to Adrenocortical Function in the Rat.* (24541)

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Certain experiments have indicated decreased steroid secretion by pyridoxine-depleted animals(1,2,3,4) while other observations led to the conclusion that adrenocortical hormone production is not impaired(5,6,7). In these earlier studies steroid secretion was determined by indirect methods and lack of precise measurement of adrenal hormone production may be responsible for the opposing views. The purpose of experiments reported here was to evaluate adrenocortical function of pyridoxine deficient rats by direct analysis of steroid secretion.

Methods. Young, male, Sprague-Dawley rats weighing 45-65 g were used. Animals were housed individually and weighed twice weekly. In each experiment there was a group fed a pyridoxine-deficient diet, a pair-fed control group, and an *ad lib.* fed control group. A synthetic pyridoxine-deficient diet complete with respect to other nutrients was prepared in this laboratory. Deficient and control groups were fed the same diet; however, control food was fortified with pyridoxine (22 mg/kg). At termination of each experiment, rats were killed by decapitation. The liver of each animal was excised and in Exp. I and III this organ was weighed. Hepatic tissue weighing approximately 400 mg was removed from left lateral lobe and homogenized in 10 volumes of phosphate buffer, pH 7.4 for 45 seconds. One ml of homogenate was diluted with 9 ml phosphate buffer and glutamic-pyruvic transaminase enzyme activity of this solution determined by the method of Karmen *et al.*(8). Protein determinations were also done on liver homogenate by the technic of Lowry(9). Adrenals were removed immediately after animals were sacrificed, dissected free of surrounding tissue, bisected and weighed. Both adrenals from each rat were placed in a beaker containing 2 ml of Krebs-Ringer-phosphate-glucose solution, pH 7.4, to which one unit of ACTH was added. Adrenals were incubated aerobically for 2 hours at 37°C. On completion of incubation, steroids secreted into the medium were recovered and quantitatively determined by method previously described(10). *Exp. I.* Animals were placed in 3 groups and given diets as described. The study was continued for 60 days. *Exp. II* was carried out according to plan except that 4-desoxypyridoxine, a pyridoxine antagonist, was added to food (30 mg/kg) given all animals. Desoxypyridoxine was used to reduce the time required to induce Vit. B_6 deficiency. Experiment was terminated after 19 days. *Exp. III.* Three groups of rats were fed diets according to plan for 62 days. At this time half the animals in each group were sacrificed. Desoxypyridoxine (30 mg/kg) was then added to food given all remaining animals and the study continued 4 additional days. Several times during the last 20 days of experiment, rats from each group were given 25 mg of dl-tryptophan orally and excretion of xanthurenic acid determined during the succeeding 24 hours(11).

Results. Exp. I. Results are summarized in Table I. Rats on Vit. B_6 deficient diet gained weight slowly for about 2 weeks. Subsequently, weight remained relatively constant. Other manifestations of deficiency appeared after 40-50 days had elapsed. At this time acrodynia was present, animals were less active than previously, and a few rats died. Pair-fed control rats gained weight more rapidly than did the deficient group and reached maximum weight after about 3 weeks. Thereafter, weight remained fairly constant, animals were active and showed no evidence of illness. *Ad lib.* fed controls gained weight steadily throughout the period and appeared

* This project was carried out with aid of research grants from National Vit. Fn. and Williams-Waterman Fund.

TABLE I. Body, Adrenal and Liver Weights, Liver Glutamic Pyruvic Transaminase Activity, and Adrenocortical Hormone Secretion of Various Groups after 2 Months on Experimental Diets (Mean Values ± Standard Error of Mean).

Group	Body wt (g)	Adrenal wt (mg/100 g body wt)	Liver wt (g/100 g body wt)	Liver transaminase activity*	Adrenal steroid secretion (μg/100 mg adrenal wt)
B_6 deficient (9)†	81.1 ± 3.5	39.9 ±3.02	3.61 ± .14	.57 ± .03‡	76.1 ± 5.8
Pair fed control (9)	116.3 ± 1.3	26.5 ± .68	2.59 ± .07	.81 ± .05	77.8 ± 3.6
Ad lib fed control (9)	315.6 ± 9.5	13.6 ± .64	3.71 ± .07	.46 ± .03	62.6 ± 2.0

* μmoles pyruvate formed/hr/100 μg protein. † No. of animals in group. ‡ Mean of 7 determinations.

well. Adrenal weights, expressed in relation to body weight, demonstrate that marked adrenal hypertrophy occurred in the pyridoxine depleted group. Differences in adrenal weight between deficient and control rats, both pair-fed and *ad lib.* fed, were highly significant, P = <0.01. Liver weight to body weight ratio was almost the same in depleted rats as in *ad lib.* fed animals but was significantly greater (P = <0.01) than that of pair-fed controls. Liver glutamic pyruvic transaminase activity was significantly reduced in the deficient group when compared to pair-fed controls (P = <0.01) but was not significantly different from that of *ad lib.* fed rats. Adrenal steroid secretion by deficient and pair-fed rats was almost the same. Although adrenal hormone secretion of *ad lib.* fed controls was less than that of the other groups, differences were not statistically significant.

Exp. II. Results are summarized in Table II. Rats which consumed the B_6 deficient diet containing desoxypyridoxine failed to gain weight. These animals had diminished appetite and acrodynia after being on diet only 7-10 days. Although the control diet contained desoxypyridoxine in the same concentration as the pyridoxine-deficient food, pair-fed and *ad lib.* fed controls gained weight and appeared well. Adrenal hypertrophy was again marked in deficient rats and differences in adrenal weight between these rats and pair-fed and *ad lib.* fed controls were highly significant, P = <0.01. Hepatic transaminase activity was essentially the same in deficient and pair-fed controls; however, activity of this enzyme in *ad lib.* controls was significantly reduced (P = <0.01). Differences in adrenal steroid secretion by the 3 groups were small and not significant.

Exp. III. Results are summarized in Tables III and IV. B_6 deficient rats showed a small weight gain during the first 3 weeks but subsequently little change in weight occurred. Other manifestations of pyridoxine depletion appeared after 45 days but were not severe until desoxypyridoxine was added to the diet (day 63). No abnormalities were noted in control animals at any time.

Adrenals of deficient rats were larger than those of controls. The difference in adrenal weight between deficient and pair-fed control rats after 62 days was significant, P = <0.05. Adrenal weights of deficient and *ad lib.* fed controls at this time differed significantly,

TABLE II. Body and Adrenal Weights, Liver Glutamic Pyruvic Transaminase Activity, and Adrenal Steroid Hormone Secretion of Various Groups after 19 Days on Diets Containing Desoxypyridoxine (Mean Value ± Standard Error of Mean).

Group	Body wt (g)	Adrenal wt (mg/100 g body wt)	Liver transaminase activity*	Adrenal steroid hormone secretion (μg/100 mg adrenal wt)
B_6 deficient (11)†	57.2 ± .66	52.5 ± 1.73	.40 ± .02	71.0 ± 7.03
Pair fed control (10)	86.7 ± 1.33	30.0 ± .87	.43 ± .02	66.7 ± 3.87
Ad lib fed control (10)	172.8 ± 4.70	19.7 ± .78	.33 ± .01	61.5 ± 2.48

* μmoles pyruvate formed/hr/100 μg protein. † No. of animals in group.

TABLE III. Body, Adrenal and Liver Weights of Various Groups (Mean Values ± Standard Error of Mean; Each Mean Represents 5 Determinations Except Where Indicated by Number in Parentheses).

Group	Body wt (g)		Adrenal wt (mg/100 g body wt)		Liver wt (g/100 g body wt)	
	A*	B*	A	B	A	B
B_6 deficient	89.1 ± 2.06	107.0 ± 8.46 (6)	35.8 ± 1.62	37.9 ± 2.32 (6)	4.29 ± .27	3.58 ± .13 (6)
Pair fed control	131.3 ± 3.01	131.2 ± 3.12	28.1 ± 2.27	25.0 ± .63	2.76 ± .18	2.28 ± .10
Ad lib fed control	313.1 ±11.3	333.9 ±23.0	15.3 ± .69	13.3 ± 1.03	3.78 ± .12	3.44 ± .13

* Values in columns labelled A are for rats fed experimental diets for 62 days; in columns labelled B for rats fed experimental diets for 62 days and were given experimental diets *plus* desoxypyridoxine for 4 additional days.

$P = <0.01$. Differences in adrenal weight of deficient and control animals on day 66 (after 4 days of consuming food containing desoxypyridoxine) were significant, $P = <0.01$. Liver weight-body weight ratios were almost the same in deficient rats and *ad lib.* controls on days 62 and 66; however, in each instance liver weight of pair-fed controls was significantly reduced ($P = <0.01$).

Liver glutamic pyruvic transaminase activity of pyridoxine-deficient rats was less than that of pair-fed controls on day 62, but this difference was not statistically significant. On day 66, after desoxypyridoxine feeding, liver transaminase activity of deficient rats was further reduced and the difference from the level in pair-fed controls was significant, $P = <0.01$. Hepatic transaminase enzyme activity of *ad lib.* controls was approximately the same as that of deficient rats at both times. Adrenocortical hormone secretion of deficient rats on days 62 and 66 was not significantly different from that of pair-fed or *ad lib.* fed controls.

Xanthurenic acid excretion of depleted rats following oral administration of 25 mg of dl-tryptophan averaged 3.2 mg per day. When control animals were given equivalent doses of tryptophan, only traces of xanthurenic acid were excreted.

Discussion. It was decided to use liver glutamic pyruvic transaminase enzyme activity as evidence of Vit. B_6 deprivation since it has been shown that activity of this enzyme is directly related to B_6 intake(12). Although transaminase activity was less in pyridoxine-deprived animals than in pair-fed controls in all experiments, differences were significant only in Exp. I and the second part of Exp. III. Furthermore, in most instances transaminase activity of deficient rats was greater than that of *ad lib.* controls. Since a marked reduction of transaminase activity did not occur in rats fed the deficient diet, the question arises as to whether B_6 deficiency was present in these animals. That deficiency did occur is evident for the following reasons. First, rats were fed a synthetic diet containing no pyridoxine and clinical manifestations of the vitamin deficiency were present. Finally, B_6

TABLE IV. Liver Glutamic Pyruvic Transaminase Activity and Adrenocortical Hormone Secretion of Various Groups (Mean Values ± Standard Error of Mean; Each Mean Represents 5 Determinations Except Where Indicated by Number in Parentheses).

Group	Liver transaminase activity*		Adrenal steroid secretion (μg/100 mg adrenal wt)	
	A†	B†	A	B
B_6 deficient	.40 ± .07	.30 ± .02(6)	70.3 ± 12.48	67.9 ± 5.39(6)
Pair fed control	.51 ± .05	.53 ± .02	80.2 ± 5.44	82.1 ± 4.29
Ad lib fed control	.35 ± .07	.34 ± .05	68.1 ± 9.75	62.4 ± 7.89

* μmoles pyruvic acid formed/hr/100 μg protein.

† Values in columns labelled A are for rats fed experimental diets for 62 days; in columns labelled B for rats fed experimental diets and were given experimental diets *plus* desoxypyridoxine for 4 additional days.

depletion was clearly demonstrated in deficient animals of Exp. III by measuring urinary excretion of xanthurenic acid following oral administration of dl-tryptophan. Animals in the depleted group excreted large quantities of xanthurenic acid following oral tryptophan whereas both pair fed and *ad lib.* fed controls excreted only minute amounts of this substance. Therefore, despite the small changes in liver glutamic pyruvic transaminase activity which occurred in depleted rats, it is apparent that Vit. B_6 deficiency was present in these animals.

Liver weight to body weight ratio of pair fed rats was significantly less than that of either the pyridoxine-deficient group or *ad lib.* fed controls. Earlier studies have shown that partial starvation results in decreased liver size hence the occurrence of smaller livers in pair-fed rats than in *ad lib.* fed animals is not surprising(14). It was not anticipated, however, that liver weight of deficient rats would be greater than that of pair-fed controls and results of this investigation do not provide an explanation for this observation. Histologic study of various organs in B_6 deficient rats conducted by other investigators has demonstrated abnormal accumulation of fat in the liver and it may be for this reason that hepatic weight was increased in depleted animals(5).

Marked adrenal enlargement was observed in experiments described herein. Such hypertrophy must occur as a result of B_6 deficiency and is not due simply to partial starvation since adrenal enlargement was greater in depleted rats than in pair-fed controls. In contrast to previous investigations, adrenocortical function was assessed in these studies by direct determination of steroid secretion. The results obtained in all experiments show that hormone production by adrenals of B_6 deficient rats was equal to that of pair-fed and *ad lib.* fed control animals. It is therefore concluded that function of the adrenal cortex is not altered in Vit. B_6 deficiency even though hypertrophy of this gland occurs.

Summary. Vit. B_6 deficiency was induced in growing rats by feeding a pyridoxine deficient diet. In certain experiments desoxypyridoxine, a metabolic antagonist of Vit. B_6, was added to shorten the period required to induce this vitamin deficiency. In each experiment pair-fed and *ad lib.* fed controls were given the same diet as consumed by deficient rats except that pyridoxine was added. After deficiency became manifest adrenocortical function of experimental animals was measured by determining steroid secretion of isolated adrenals in response to ACTH. The results show that while marked adrenal hypertrophy occurs in B_6 deficient rats adrenocortical hormone secretion is not impaired.

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Amino Acid Requirements of the Novikoff Hepatoma *in vitro*. (24542)

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Amino acid requirements of a number of mammalian cell strains have been determined under tissue culture conditions. These not only included some serially-propagated cells of human origin(1,2) but also neoplasms of rat(3) and mouse(4), monkey testicular and kidney cells(5,6), and rabbit fibroblast(7). In general, the essential amino acids seemed to follow a similar pattern, *i.e.*, the 8 essential amino acids (lysine, leucine, isoleucine, threonine, valine, phenylalanine, tryptophan, and methionine) as well as arginine, histidine, tyrosine, cysteine, and glutamine. The present report describes experiments designed to determine the amino acids necessary for growth and survival of the Novikoff hepatoma *in vitro*.

Methods. The stock tumor* was carried intraperitoneally in Holtzman rats. Six days after tumor transplantation, the animals were sacrificed and initial inoculum for tissue culture was prepared from freshly excised tumor. Details of experimental technics have been described(3,10), and basal medium 5a was used. This medium was the same as previously reported(11) except that dialyzed pooled human serum was used in lieu of bovine serum, all amino acids were the L-isomer, L-serine included in substrate at a concentration of 0.25 mM, and i-inositol was added at 36.0 μg/ml final medium. Since the pH of substrate was a vital factor in growth of Novikoff hepatoma *in vitro*(12), the flasks were plugged with gauze stoppers and incubated in a flowing atmosphere of 7% CO_2 in air. The initial inoculum was 10,000 cells/T-15 flask (total volume 2 ml), and growth response was determined by a whole cell count.

Results. The effect of omitting individual amino acids from the medium can be seen in Table I. It was apparent that the 13 amino acids (including glutamine) previously reported to be required by a number of cell lines (1,2,4) were essential for growth of Novikoff hepatoma *in vitro*. The essentiality of glycine was not clear-cut, however, since growth was observed, but it was obviously inferior to that obtained in a complete medium. When the principal source of amino acid nitrogen was restricted to contain only 13 essential amino acids, only a cell maintenance condition was observed during 8-day incubation period. Some growth was obtained by adding 0.25 mM serine to the restricted medium, but supplementing with 0.1 mM glycine resulted in a 56-fold increase in cells during this time. When both glycine and serine were added, growth was comparable to a complete medium containing the 21 amino acids and amides in basal medium 5a (Fig. 1). From this, it was apparent that glycine and serine definitely stimulated growth of Novikoff hepatoma, and glycine was more effective than serine.

While amino acid requirements of several cell lines cultured *in vitro* seemed to follow a general trend, further investigations into this area revealed many interesting differences. For example, Walker tumor required asparagine(8), rabbit fibroblast, serine(7), and monkey testicular cell, glycine(5) in addition to the 13 amino acids or amides reported essential for HeLa tumor(1) and Strain L(4). Further, chick heart fibroblast did not require glutamine or isoleucine; and alanine, aspartic

TABLE I. Effect of Omitting Individual Amino Acids from Substrate on Growth of Freshly Excised Novikoff Hepatoma.

Amino acid	Growth* in 8 days	Amino acid	Growth* in 8 days
Control	24.0	Phenylalanine	1.8
Arginine	4.2	Tyrosine	1.8
Histidine	1.6	Tryptophan	.2
Lysine	.9	Cysteine	0
Glycine	8.4	Methionine	.9
Alanine	24.9	Glutamine	.4
Serine	28.9	Asparagine	34.6
Threonine	.4	Aspartic acid	30.0
Valine	.9	Glutamic acid	33.5
Leucine	1.1	Proline	31.5
Isoleucine	.2	Hydroxyproline	29.5

* Initial inoculum referred to as 1.

* Kindly supplied by Dr. Alan C. Sartorelli, McArdle Memorial Laboratory, Madison, Wis.

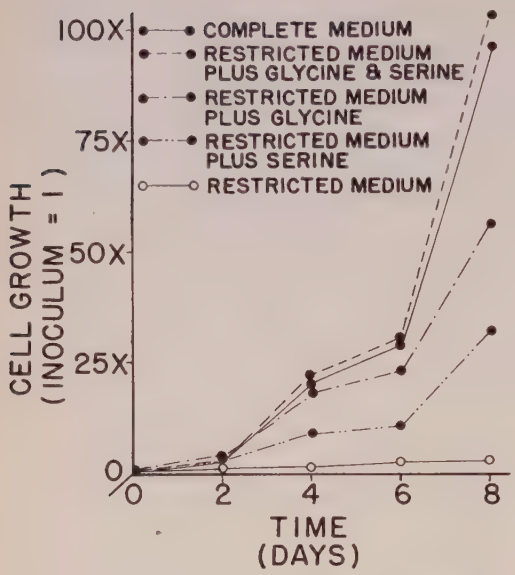

FIG. 1. Growth response of Novikoff hepatoma to glycine and serine. Amino acids in the restricted medium were only those essential for growth.

acid, glutamic acid, proline, and hydroxyproline inhibited growth of this cell line(9). In the present studies, no growth was obtained when both glycine and serine were deleted from the substrate, suggesting that one (possibly glycine) was essential for growth, but the presence of serine in the substrate could partially spare this requirement.

Further metabolic differences can be distinguished between cell lines if one would consider the results of sparing experiments. Attempts to spare the tyrosine requirement of Novikoff cells by increasing amounts of phenylalanine yielded negative results. However, glutamine, cysteine, and arginine requirements could be spared (Table II). While glutamic acid at extremely high concentrations (20 mM) was toxic, it could partially spare the glutamine requirement at intermediate levels (5-10 mM). Glutamic acid also could partially spare the glutamine requirement for monkey testicular cell(5), while glutamic acid and glutamine were readily interconvertible in monkey kidney cell(6). Further, aspartic acid and asparagine spared the glutamine requirement for monkey kidney cells, but these compounds were ineffective for Novikoff hepatoma (Table II) or Walker tumor(8).

The requirement of cysteine for Novikoff cells was alleviated with moderate amounts of sodium thioglycolate, as well as sodium sulfite, sodium bisulfite, sodium thiosulfate, and sodium sulfide, but methionine, cysteic acid, or sodium sulfate was not effective. Combinations of the inorganic substances with further supplementation of serine did not improve cellular growth markedly. Similar evidence for sparing cysteine requirement for a number of cell lines was reported by Eagle(6) but chick heart fibroblast has shown an absolute requirement for cysteine or cystine(13,14).

Of particular interest were the arginine-sparing experiments. While ornithine was ineffective, supplementing an arginine-deficient

TABLE II. Some Typical Sparing Experiments with Freshly Excised Novikoff Hepatoma *In Vitro*.

		Concentration (mM)					
		0	.5	1	5	10	20
Deficiency	Supplement	Growth in 8 days, inoculum = 1					
Glutamine	Glutamic acid	.7	1.6	1.6	5.3	6.7	.2
	Aspartic "	.4	.7	.4	.0	.4	.0
	Asparagine	.4	.2	.0	1.6	.8	.4
Cysteine	Methionine	.2	.4	.2	.4	.0	.9
	Sodium thioglycolate	.9	12.0	11.3	8.2	5.3	2.2
	Cysteic acid	.2	0	.0	.0	.0	.0
	Na_2SO_4	.2	.4	.2	.2	.0	.0
	Na_2SO_3	.0	12.0	8.4	1.6	.4	.0
	$NaHSO_3$	.0	8.9	10.4	3.3	.4	.4
	$Na_2S_2O_3$	.2	1.1	9.3	10.4	6.2	8.8
	Na_2S	.2	.4	.6	10.9	9.6	7.8
Arginine	Citrulline	1.6	5.3	13.5	47.7	42.6	49.1
	Ornithine	1.6	2.9	1.8	.2	.7	.2

Control growth range = 86.5-90.4

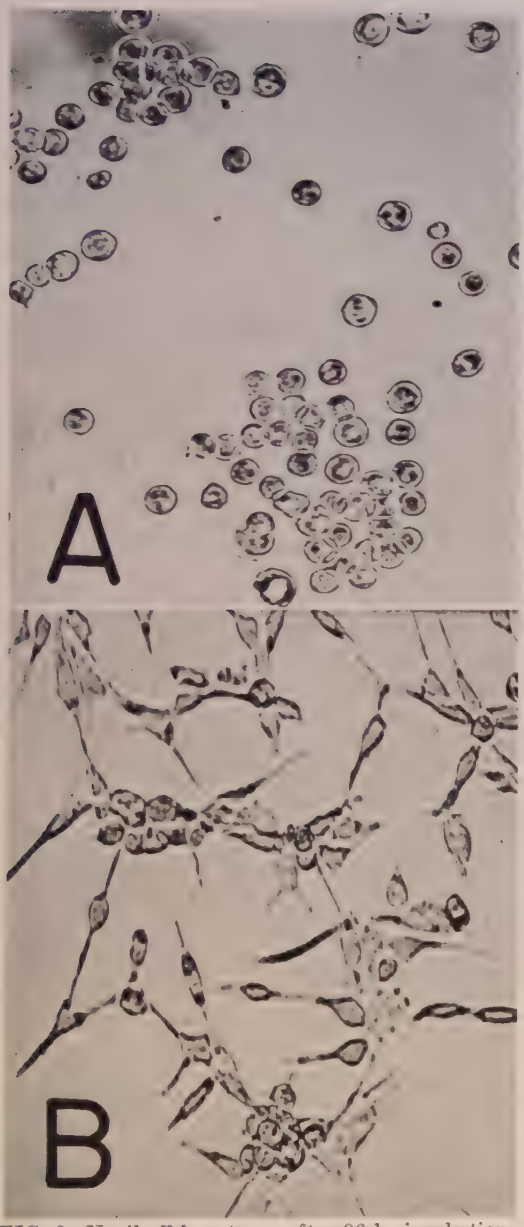

FIG. 2. Novikoff hepatoma after 96 hr incubation. A—complete medium 5a; B—complete medium 5a – arginine. 240×.

medium with citrulline resulted in excellent growth of Novikoff hepatoma *in vitro*. At the highest level of citrulline tested (20 mM), a 49-fold increase in cells was obtained during the 8-day incubation period. Since this neoplasm arose from hepatic tissue, it appeared that a part of metabolic sequences of the Krebs-Henseleit cycle was still operating in the resultant tumor. Citrulline was previously reported to be inactive in replacing arginine for Walker tumor *in vitro*(15), but Morgan(16) has shown it to be active for chick heart fibroblast.

Some mention should be made of the morphological appearance of Novikoff cells in arginine-deficient medium. In a complete medium the cells were rounded, non-granular in appearance, with many mitotic figures, but adhesion to the glass surface was poor. Many cells exhibited balloon-like extensions of the cell surface, previously noted by Hotchin(12). As the growth period was extended, numerous giant cells appeared with granulated cytoplasm and enlarged nuclei. When arginine was omitted from the substrate, the rounded cells were no longer present; and the culture consisted almost entirely of fibroblasts or spindle cells (Fig. 2). They were less refractile and practically all cells adhered to the surface of glass vessel. Pathological examination indicated that these cells were neoplastic in nature and were not derived from normal stroma of the tumor.† Furthermore, this phenomena appeared to be reversible. For example, when cells cultured in an arginine deficient medium for 4 days were placed in a complete medium, they became rounded, many mitotic figures were apparent, and cell population increased. Attempts, however, continuously to culture fibroblasts for further studies failed, since the cells would usually die after 6-10 days in an arginine-deficient medium. Relation of arginine to morphology of Novikoff hepatoma cells *in vitro* merits further investigation.

Summary. Novikoff hepatoma requires 12 amino acids and glutamine for growth *in vitro*. When both glycine and serine were deleted from the medium, no growth occurred. In presence of serine some growth was apparent, but glycine was more effective in stimulating tumor cell proliferation. Glutamic acid and sodium thioglycolate could partially spare glutamine and cysteine requirements, respectively, and several sulfur-containing inorganic salts could partially replace cysteine. Argi-

† We are indebted to Dr. E. S. Irvine for pathological examinations.

nine could be replaced by citrulline but ornithine was not active. When arginine was deleted, the typically rounded cell of Novikoff hepatoma was no longer apparent; but a predominance of fibroblasts remained attached to the surface of the flasks.

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Creatine Metabolism in Hyperthyroidism.* (24543)

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Present knowledge of the influence of hyperthyroidism on creatine metabolism has been summarized(1). It was pointed out that the disease leads to an elevated creatinuria and reduction in concentration of muscle creatine. Several possible explanations for this metabolic defect were suggested although no direct experimental evidence supported them. The present report presents the results of a study of creatine metabolism in hyperthyroidism utilizing radioactive creatine precursors in a manner similar to that employed in a study of creatine metabolism in Vit. E deficiency (3).

Methods. Two series of experiments were conducted. In the first, Sprague-Dawley rats of both sexes, weighing initially between 80 and 100 g were given a diet of laboratory chow. Four rats were given daily subcutaneous injections of 0.5 mg of sodium thyroxine. Four other rats were given no injections and served as controls. After 3 weeks the rats were each injected intraperitoneally with 100 microcuries of glycine-1-C^{14} (specific activity 0.81 microcuries per μmole) per kilo of body weight. The animals were killed 2 or 3 hours after the injection. Since there appeared to be no significant difference in results obtained from animals killed 2 hours after glycine injection as compared to those killed after 3 hours, the results were combined. Glycocyamine and creatine concentrations and specific activities were determined as previously described(3). In the second series of experiments, weanling Sprague-Dawley rats of both sexes were given a purified diet consisting of casein, 18 g; sucrose, 74.5 g; hydrogenated vegetable fat (Crisco), 3 g; cod liver oil, 2 g; salt mix(2), 2 g; choline chloride, 0.1 g; thiamine chloride, 1.5 mg; riboflavin, 1.5 mg; niacin, 6 mg; inositol, 30 mg; calcium pantothenate, 3 mg; pyridoxine hydrochloride, 1.5 mg; biotin, 15 μg; 2 methyl 1, 4 naphthoquinone, 75 μg; Vit. B_{12}, 10 μg. Each rat was given 10 mg alpha

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TABLE I. Some Metabolic Effects of Hyperthyroidism of Rats.

Animals	Avg wt gain	Avg food intake	Urinary excretion, mg/100 g body wt /day		Organ wt, mg/g body wt		Heart creatine	Skeletal muscle creatine	Kidney glycocyamine
	g/day		Creatinine	Creatine	Heart	Kidney	mg/100 g		
Normal	3.9	9.2	4.61	.52	4.3	8.0	222	389	8.61
Hyperthyroid	2.9	8.7	5.33	.84	8.8	11.3	115	349	13.95
P value*	<.001	<.3	<.025	<.001	<.001	<.4	<.001	<.20	<.10

* Probability that differences observed are due to chance.

tocopherol 2 times weekly. One group was given this diet without supplement and will be referred to as normal while a second group was given in addition 2.0% desiccated thyroid (Nutritional Biochemicals Corp.). After 2 weeks' feeding, the animals receiving the desiccated thyroid exhibited the usual signs of hyperthyroidism. Four animals from each group were then injected with glycine-1-C^{14} at the same dosage as used for series 1. Two animals from each group were killed 30 minutes after glycine injections and 2 after one hour. Glycocyamine and creatine concentrations and specific activities were determined as previously described(3). Skeletal muscle protein specific activity was determined after repeated precipitation with trichloroacetic acid. Eight animals from each group in the second series were injected intraperitoneally with 0.5 microcuries of creatine-1-C^{14} (specific activity 10 microcuries per mg) per 100 g body weight. Within each group 3 animals were killed one hour after creatine injection, 3 after 2 hours and 2 after 4 hours. Blood was collected at this time and the C^{14} counts of the serum were measured. Skeletal muscle and heart creatine were then isolated for counting(3). During the last day of the feeding period animals were placed in metabolism cages and urine was collected for creatine and creatinine determination(4). The data were analyzed for statistical significance by the t-test(5).

Results. Specific activities of kidney glycocyamine and skeletal muscle creatine from hyperthyroid rats fed chow diet and injected with glycine-1-C^{14} were reduced to approximately ¼ the values obtained with normal rats. The reduced specific activity of kidney glycocyamine in the hyperthyroid rats may be related to the time interval between glycine injection and sacrifice of the animals. Other experiments have indicated that kidney glycocyamine specific activity reaches a peak within a few minutes after glycine injection. In spite of the reduced specific activity of kidney glycocyamine, liver creatine from hyperthyroid rats exhibited an elevated specific activity. These results suggest that rate of creatine synthesis is increased in hyperthyroidism. The reduced specific activity of skeletal muscle creatine from hyperthyroid rats indicates that in hyperthyroidism rate of entry of creatine into skeletal muscle is depressed.

The data in Tables I through III were obtained in the second series of experiments. The hyperthyroid animals gained less weight although food intake was not significantly reduced. Creatine excretion was elevated in the hyperthyroid rats as would be expected; creatinine excretion was unaffected. There was a striking increase in heart weight of the hyperthyroid rats with a decrease in concentration of heart creatine. Skeletal muscle creatine concentration was only slightly reduced.

The data in Table II confirm the findings made in the first series of experiments. The results indicate that hyperthyroidism in the rat leads to an elevated rate of creatine synthesis with reduction in incorporation of creatine into skeletal muscle. Incorporation of creatine into cardiac muscle was also depressed in hyperthyroid rats. Hyperthyroidism led to an increased incorporation of glycine into skeletal muscle protein. This result indicates an elevated rate of protein turnover in muscle of hyperthyroid rats which may explain the well-documented increase in uri-

TABLE II. Incorporation of Glycine-1-C^{14} into Protein, Glycocyamine and Creatine by Normal and Hyperthyroid Rats.

Interval after inj., hr	Animals	Kidney glycocyamine	Liver creatine	Skeletal muscle creatine	Heart creatine	Skeletal muscle protein, c.p.m./mg
		c.p.m./μmole				
½	Normal	1697	57	19	66	7.2
½	Hyperthyroid	656	64	13	38	11.2
1	Normal	1397	27	27	88	15.3
1	Hyperthyroid	551	36	15	48	18.3

nary nitrogen in this disease(1).

The data obtained from creatine-C^{14} injections are given in Table III. Serum creatine-C^{14} was considerably elevated in the hyperthyroid rats at all time intervals. There was essentially no effect of hyperthyroidism on specific activity of muscle creatine. If muscle creatine specific activity is divided by serum creatine-C^{14}, which presumably is the immediate precursor of muscle creatine, then rate of incorporation of creatine into muscle would appear to be considerably reduced in the hyperthyroid rats.

From the combined results of these experiments it appears that in hyperthyroidism the rate of synthesis of creatine is elevated while incorporation of creatine into skeletal muscle and cardiac muscle is considerably depressed. This situation may be contrasted with Vit. E deficiency in which the creatinuria is due to an inability of muscle to retain creatine after its incorporation. In the latter condition the entry of creatine into the muscle proceeds at a greatly increased rate(3). Thus the creatinuria of hyperthyroidism and Vit. E deficiency are the result of fundamentally different metabolic lesions.

TABLE III. Metabolism of Creatine-1-C^{14} by Normal and Hyperthyroid Rats.

Interval after inj., hr	Animals	Serum C^{14}, c.p.m./ml	Skeletal muscle creatine	Heart creatine
			c.p.m./μmole	
1	Normal	20	82	91
1	Hyperthyroid	87	76	109
2	Normal	23	92	110
2	Hyperthyroid	45	101	107
4	Normal	13	120	125
4	Hyperthyroid	35	119	188

Summary. Hyperthyroidism was induced in rats by thyroxine injections and by feeding of desiccated thyroid. Normal and hyperthyroid rats were injected with glycine-1-C^{14} and incorporation of the isotope into glycocyamine and creatine was determined. In other experiments normal and hyperthyroid rats were injected with creatine-1-C^{14} and its incorporation into skeletal muscle and heart creatine was determined. Hyperthyroidism resulted in an increased creatinuria, and increased heart weight and a decreased heart creatine concentration. Hyperthyroidism led to increased incorporation of glycine into liver creatine and to decreased incorporation into skeletal muscle and heart creatine. It is concluded that the creatinuria of hyperthyroidism is the result of a block in incorporation of creatine into skeletal muscle and cardiac muscle.

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Aromatic Amines* III. Note on Bis(2-amino-1-naphthyl) Phosphate, a Urinary Metabolite of 2-naphthylamine. (24544)

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Recent emphasis on the study of 2-naphthylamine in relation to its ability to produce bladder cancer in man and dog has been on metabolic formation of the active carcinogen. The metabolically formed carcinogenic derivative presumably appears in the urine from the inactive primary compound and gives rise to tumor formation in the bladder. Within this concept, identificaton of this carcinogenic metabolite is an initial obligation for understanding of the etiology of this disease. Earlier papers in this series(1,2) have described some excreted metabolites of aromatic amines; the present paper is concerned with demonstration of a novel metabolite, a phosphate conjugate of 2-amino-1-naphthol in the urine of dogs. This compound appears to be a promising candidate for the role of the active bladder carcinogen.

Methods. Our experiments were carried out by feeding 1 g of 2-naphthylamine per day to 3 dogs and observing the urinary metabolites of this compound. Total aromatic amine excretion was measured by the specific method employing 1,2-naphthoquinone-4-sulfonate (NQS)(1) and 2-amino-1-naphthol was determined by the color test described by Clayson(3). Our attention was drawn to the fact that 2-amino-1-naphthol appeared in increased concentration in alkaline urines. This pointed to an alkali labile precursor of 2-amino-1-naphthol. The known conjugates of 2-amino-1-naphthol, the glucuronide and the sulfate esters, both are stable in alkali. Furthermore it has been reported that no enzyme capable of splitting sulfate esters of ortho amino phenols exists in urine(4) and that the glucuronidases present have a pH optimum at pH 4.5(5). The alkali labile conjugate was readily extractable by ether and was water soluble. Chromatography with n-amyl alcohol revealed a single NQS reacting spot with an R_F of 0.8. The compound was isolated as the amine hydrochloride by passing hydrochloric acid gas through a dry ether solution. The material yielded ortho phosphate and oxidation products of 2-amino-1-naphthol on hydrolysis with strong acid, or by acid and alkaline phosphatases. The hydrolysis with acid phosphatase proceeds in 2 stages—in the first stage, one mole of 2-amino-1-naphthol was released and no ortho phosphate; in the second, another mole of amino-naphthol was released and one mole ortho phosphate (Table I). The ratio of amine to phosphate was two to one. We interpret this as evidence that we are deal-

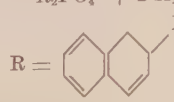
TABLE I. Acid Phosphatase Action on Metabolite. 0.112 μm amine (NQS) of metabolite in 10 ml 0.1M 5.2 acetate buffer was incubated with 10 mg acid phosphatase (Worthington Corp.) at 37°C. After 5 min. and 17 hr 2-amino-1-naphthol was determined by method of Clayson(3) and phosphate by method of Lowry and Lopez(6).

Time	2-amino-1-naphthol (μm) Found	Expected*	Phosphate (μm) Found	Expected*	Amine (NQS) phosphate ratio Found	Expected*
5 min.	.069	.056	.005	0		
17 hr	.120	.112	.055	.056	2	2

* Expected values are calculated for the following reactions.

5 min. $R_2PO_4^- + H_2O \rightarrow ROH + RHPO_4^-$

17 hr $R_2PO_4^- + 2\,H_2O \rightarrow 2\,ROH + H_2PO_4^-$



* Aided by grant from Allied Chemical Corp.

ing with a bis(2-amino-1-naphthyl) phosphate. In the first stage, one mole of 2-amino-1-naphthol is released plus mono-aryl phosphate and in the second, the mono-aryl phosphate is hydrolyzed.

Phosphate conjugates have not been described previously as end products of metabolism of foreign compounds. Three features make this compound a promising candidate for the active bladder carcinogen: 1. The compound is readily split by phosphatases occurring in the dog urine to form 2-amino-1-naphthol which has been shown to be an active carcinogen(7). 2. The compound has solubility properties characteristic of compounds capable of crossing the cell wall. 3. Preliminary observations in rabbits injected with 2-naphthylamine indicate the absence of similar phosphate conjugates. Boyland and Manson(8) report the absence of 2-amino-1-naphthyl phosphate in the rat. Both these species are relatively insensitive to bladder cancer when exposed to 2-naphthylamine, while the dog, which elaborates this phosphate conjugate, is sensitive to the disease.

Summary. Bis(2-amino-1-naphthyl) phosphate has been demonstrated as a urinary end product of 2-naphthylamine metabolism. Phosphate conjugates have not been observed previously as urinary end products. The compound may be related to the occurrence of bladder cancer in this species upon ingestion of 2-naphthylamine.

The contribution of Miss Joan Royce in this work is gratefully acknowledged.

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Ocular Transplants of Joint as an Experimental Method.* (24545)

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An experimental polyarthritis can be produced in rats and mice using species-specific strains of *Micoplasma arthritidis* (pleuropneumonia-like organism) in broth culture administered either intravenously or intraperitoneally. This experimental infection in rodents has served as a method for evaluating the effect of various environmental conditions and chemotherapeutic agents(1). It has been possible to segregate drugs into 3 groups: 1) those which prevent or cure the arthritis; 2) those which worsen its course; and 3) those without apparent effect. Because of the tendency for joint involvement to characterize this experimental infection, we wondered whether joint involvement would also appear in transplanted joint tissues. To approach this problem we adapted a technic previously employed for transplanting tumor(2) and ovarian tissues(3) to the anterior chamber of the rat eye. The first experiment was an effort to explore the possibility of intra-species (homologous) transplantation of rat joint tissues to the anterior chamber and to determine the possibility of "arthritic" changes in the transplanted tissue following intraperitoneal infection with broth cultures of *Micoplasma arthritidis* (pleuropneumonia-like organism). If "arthritic" changes could be produced in the

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TABLE I. Homologous Transplantation of Joint Tissues to the Anterior Chamber.

Exp.	Donor	Tissue	Recipient	No. of rats*	Results
1	17-day-old embryo	knee joint	mature‡	5	5/5 takes. Vascularized, grew to fill chamber by 11 days after transplantation.
2	newborn	*Idem*	"	10	10/10 takes. Vascularized, grew to fill ⅔ of chamber by 39 days after transplantation.
3	immature†	synovia and cartilage from knee	immature	7	7/7 takes. Vascularized, no growth. Sacrificed 21 days after transplantation.
4	mature	*Idem*	mature	6	6/6 no takes. Only few fine vessels which degenerated 10 days after transplantation. Tissue became fibrous.

* Transplants were made to both eyes. † 45-day-old. ‡ 120-day-old.

transplanted tissue, such changes could be readily observed through the cornea and might thus serve to indicate the effect of various drugs (Table I). In the second experiment, the recipient rats with ocular transplants of joint tissues were infected and medicated with various drugs for chemotherapeutic study (Table II).

Materials and method. Transplantation was done with entire knee joints minus the skin from both 17-day-old embryos and newborn rats. The donor animal was sacrificed, the knee joint excised, skinned, and placed in physiological saline. Synovial tissue with adhering cartilage from knee joints was also taken from immature, young adult and mature rats for transplantation. The recipient animal was anesthetized with ether and the tissue was transplanted by Goodman's technic (4) into both eyes. Tissues taken from male

TABLE II. Joint Transplantation for Chemotherapeutic Study.

Group	No. of animals	Donor	Tissue	Recipient	Treatment	Results
I	12	immature*	synovia and cartilage from knee	immature	3 controls	NCT§
					3 infected (3 days after transplantation).	⅔ rat arthritic. NCT
					3 infected (3 days after transplantation) and medicated with 0.5% phenylbutazone in ground food (Butazolidin®).	⅓ rat arthritic. NCT
					3 uninfected and medicated with 0.5% phenylbutazone in ground food.	NCT
II	10	"	*Idem*	young adult†	3 controls	NCT
					3 infected (46 days after transplantation).	
III	12	"	"	mature‡	2 control	NCT
					2 infected (2 days after transplantation) and treated with gold thioglucose in sesame oil (Solganol B®) 1 day prior to transplantation.	½ rat arthritic. NCT
					2 infected (2 days after transplantation).	½ rat arthritic. NCT
IV	9	young adult	"	young adult	3 controls	NCT
					3 broth controls	NCT
					3 infected (8 days after transplantation).	⅓ rat arthritic. NCT

* 45-day-old. † 90-day-old. ‡ 120-day-old. § No change in transplant.

donors were transplanted to male recipients and tissues taken from female donors were transplanted to female recipients. All rats used in our experiments were of the Wistar strain.

Results. Observations were made through the cornea every 3 days and histologic preparations were made of the transplants excised at various times after insertion into the anterior chamber. The tissues were immediately placed in 10% formalin after excision and stained with Meyer's haematoxylin and eosin. Criterion of viability ("takes") was based on growth and differentiation, vascularization, and maintenance for 15 days or longer without becoming fibrous or resorbed. All transplants that did not "take" due to technical error have been omitted from the data.

The results as given in Table I indicate that intraspecies (homologous) transplants were successful with the best results obtained with embryonic and newborn tissues, which grew rapidly to fill the chamber. The older tissues tend toward fibrosis and degeneration. Immature tissues failed to grow, but became vascularized and did not become fibrotic during the various experiments.

In spite of good development of polyarticular arthritis in rats infected intraperitoneally with broth cultures of *Micoplasma arthritidis* (pleuropneumonia-like organism), the tissues transplanted to the anterior chamber of these infected animals did not show gross changes characteristic of the rat polyarthritis. The use of various medications did not alter the situation, although in previous experiments many drugs have either worsened, prevented, cured, or have not changed the course of the rat disorder (Table II). The histologic sections did not enable us to grade the inflammatory cellular reaction due to infection and due to transplantation. In 2 animals with transplants and infected with *Micoplasma arthritidis* (pleuropneumonia-like organism), we have obtained negative cultures of the anterior chamber fluid.

Discussion. Thus far, it would appear that there is a "barrier" in the anterior chamber, supporting Greene(1). Also, the anterior chamber may lack something necessary to cause changes characteristic of the rat polyarthritis. Perhaps the presence of synovial tissue and the *Micoplasma arthritidis* (pleuropneumonia-like organism) may not be enough. There may be a third factor involved in causing rat arthritis. For example, the aqueous in the anterior chamber may not support growth of *Micoplasma arthritidis* (pleuropneumonia-like organism); we may not have kept the tissues in the eye long enough to adjust to the new environment before infection. These questions and problems are presently under study in our laboratory. We have studied in one group of animals the protein pattern by electrophoresis and found the beta globulin elevated in the infected animals.

Summary. 1) Intra-species (homologous) rat joint tissue transplants were successful. 2) Production of arthritis by intraperitoneal injection of broth culture of *Micoplasma arthritidis* (pleuropneumonia-like organism) did not produce gross or microscopic evidence of arthritis in the transplanted joint tissues although the recipient animal developed a good polyarticular arthritis. 3) The use of gold thio-glucose and phenylbutazone in limited numbers of infected rats did not alter the above situation. 4) Failure of transplanted joint tissues to become arthritic in infected animals suggests an ocular barrier.

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A New Chamber for Tissue Culture.* (24546)

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Carrell(1) was one of the first to recognize the necessity for apparatus which would permit detailed study of cells grown *in vitro*. Porter(2) developed a flask by flattening 2 sides of a roller tube. Many workers have made use of the hanging drop method. Rose (3) devised a chamber which permits continuous cultivation and observation of cells *in vitro*. Several tissue culture chambers made of acrylic plastic and glass have been described, among others by Christiansen *et al.* (4), Pulvertaft *et al.*(5), and more recently by Toy *et al.*(6). These chambers vary in complexity, are difficult to assemble and to sterilize, are prone to breakage and contamination. The chamber to be described overcomes these difficulties, may be used for observation of cultures at high magnifications by phase microscopy and is suitable for perfusion studies.

Description of chamber. The chamber consists of 5 parts, 2 of metal, 2 of glass and one of rubber. The metal parts may be stainless steel, or brass which is subsequently heavily chrome plated. Aluminum is unsuitable, as binding of threads readily occurs after repeated use. Price considerations make brass the metal of choice. The rubber used is a red silicone rubber "O" ring (American Packing and Gasket Co., Style DC-250, Size 6227-15). Other types of "O" rings made of various kinds of silicone and gum rubber have been found unsuitable. The remaining 2 parts are glass cover-slips of 1 inch diameter and thickness #1 or #2 (Corning Glass Co.). Cover-slips and silicone rubber are the only parts of the chamber which come in contact with the culture or nutrient, and are non-toxic. One metal part, which constitutes the bottom, has 4 holes for insertion of hypodermic needles used in filling and emptying the chamber. A drawing of chamber, with capacity of 0.7 ml, is shown in Fig. 1. It is assembled by placing a cover-slip in the metal bottom, followed by an "O" ring, on top of which is placed another cover-glass and the metal top. The top part is then screwed down with a small stirrup wrench and tightened sufficiently to prevent slippage of the "O" ring when a #22 hypodermic needle is inserted through one of the holes in the base. Degree of tightness is determined by experience of operator and thickness of cover-slip. The chamber may be sterilized in various ways. It may be sterilized by placing the component parts in a 60 mm Petri dish, and autoclaving. This method of sterilization permits the chamber to be used for observation of primary outgrowth from tissue fragments which may be placed on the lower cover glass during assembly. Or, the parts may be sterilized by flaming after dipping in 95% ethanol. Starting with the base, each part is placed after flaming in a sterile 60 mm Petri dish. When the second cover-slip has been placed on the "O" ring, the chamber may be taken out of the dish and the top, sterilized in similar fashion, screwed into place.

Procedure. After assembly, the chamber is easily filled with nutrient or cell suspension. A sterile #22 hypodermic needle is inserted through one of the ports and pushed through the silicone rubber "O" ring by gentle pressure and repeated semi-rotation; this needle serves as air vent. The cell suspension or medium is then introduced into the chamber from a Luer-Lok hypodermic syringe fitted with a #22 needle which is drilled through the "O" ring at the port directly opposite the vent needle. During filling, the chamber is held above the syringe to release entrapped air-bubbles. The vent needle is first withdrawn, then the needle used for filling the chamber. Medium changes are made in a similar manner; repeated changes of nutrient over many

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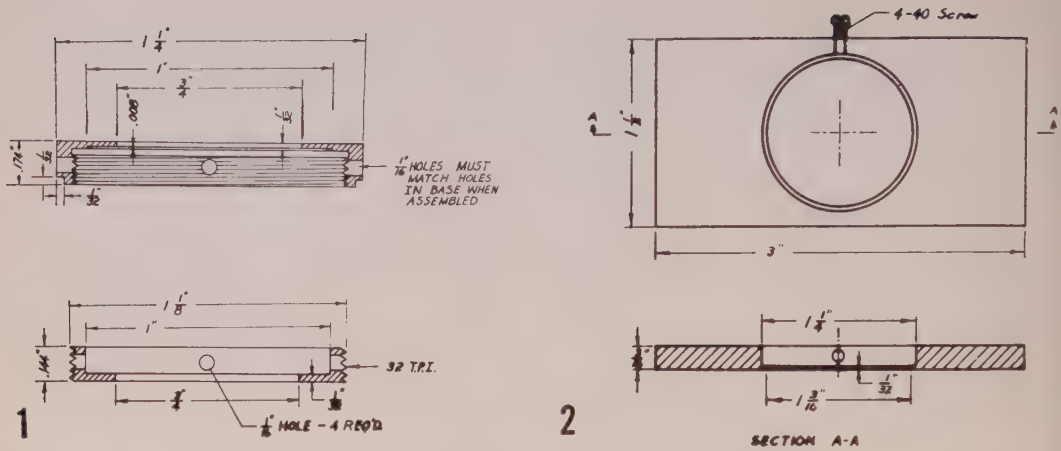

FIG. 1. Drawing showing construction of tissue culture chamber.
FIG. 2. Drawing showing construction of chamber carrier for microscopy.

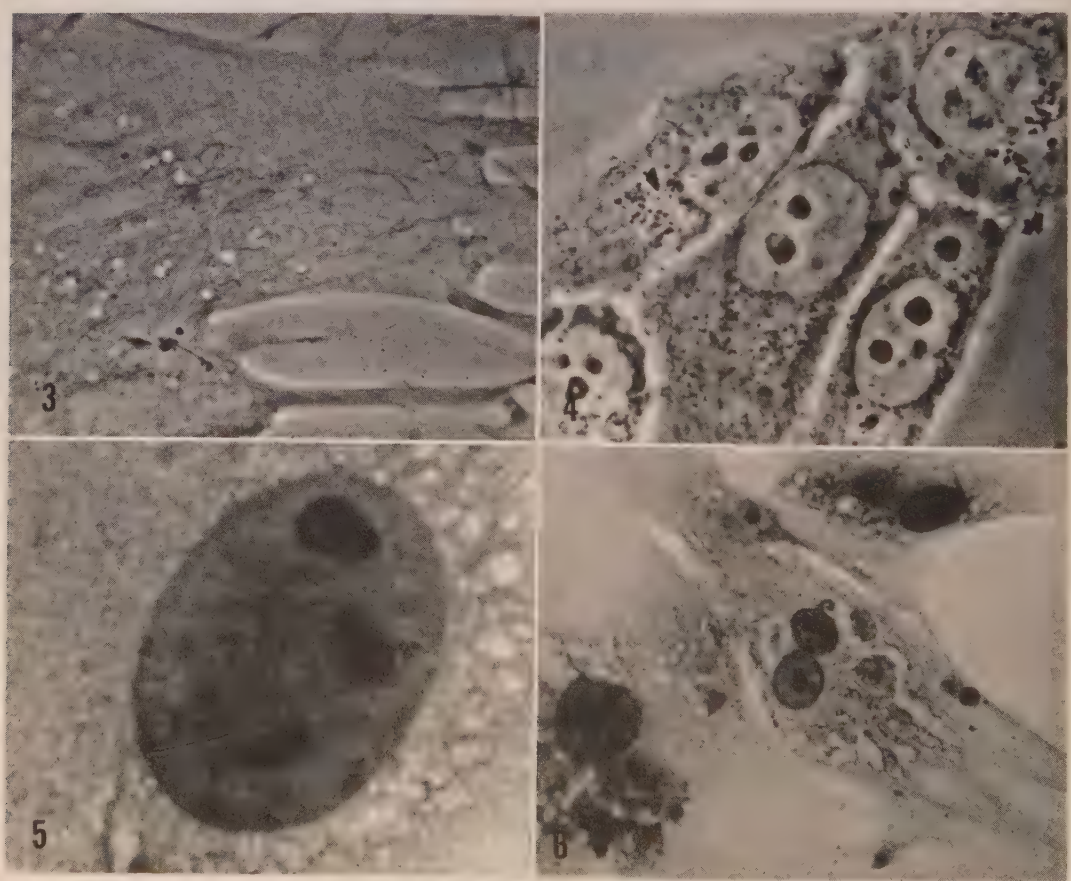

FIG. 3. Phase photomicrograph of part of a cell derived from plaque, a precursor lesion of bovine ocular squamous carcinoma, showing mitochondria. × 1200.
FIG. 4. Phase photomicrograph of human amnion cells. × 1200.
FIG. 5. Photomicrograph of nucleus of a cell derived from plaque, a precursor lesion of bovine ocular squamous carcinoma, after staining and mounting of chamber culture. × 1200.
FIG. 6. Photomicrograph showing inclusion-like bodies in cells derived from plaque. × 270.

days are possible without contamination. For culture identification and for protection of cover-slips the chamber is incubated in a 60 mm Petri dish, with the culture cover-slip at the bottom. Sub-cultures of cells growing in the chamber are made by trypsinization. Examples of cells examined by phase microscopy in the chamber are shown in Figs. 3 and 4; similar cells, fixed and stained, are shown in Figs. 5 and 6. For perfusion experiments, it is essential that the bottle used for collection of effluent be effectively plugged against aerial contaminants. For microscopy, the chamber is placed in a small rectangular carrier which will fit the slide clips of any microscope stage (Fig. 2). The carrier is provided with a locking screw which is useful in perfusion experiments where the position of the observed area must not change. Cell staining may be carried out either *in situ* or after removal of the cover slip on which the cells are growing, which is the method of choice.

Discussion. A tissue culture chamber is described which is simple in construction and use. It consists of 5 parts, 3 of these are standard items readily available. The parts of the chamber in contact with cells or nutrient are non-toxic. The apparatus is suitable for examination of cultivated cells at high magnifications by phase or bright-field microscopy. Cultures can be maintained for long periods of time in the chamber and sub-cultures are readily made. The chamber has proved valuable in tissue culture studies of material derived from cases of human leukemia, mouse leukemia, mammary carcinoma of mice, and bovine ocular squamous cell carcinoma and its benign precursor lesions.

Summary. A simple tissue culture chamber is described which permits phase microscopy at high magnifications and easy staining for permanent record. The chamber is also suited to perfusion studies, permits observation of cells over extended periods, and is useful for time lapse photography.

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Survival Time of Endotoxin Treated Rats as Response Metameter for Corticotropin and Hydrocortisone Bioassays.* (24547)

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The protective effect (survival) of adrenal cortical steroids to lethal doses of typhoid endotoxin in adrenalectomized rats(1) has been suggested as the basis of assay method for adrenal cortical steroids(2), but no detailed study of the method has been published. A protective effect has been shown in normal rats, mice, and rabbits against a variety of endotoxin preparations(3,4,5) by such materials as cortisone, hydrocortisone, corticosterone, adrenal cortical extract, and corticotropin. Desoxycorticosterone acetate was without effect. Halberg, Spink, and Bittner (6) showed that cortisone, hydrocortisone, 9-α-flurohydrocortisone, and their acetates, and to some extent aldosterone, prevented hypothermia which precedes death and prolonged survival time of adrenalectomized mice treated with Brucella somatic antigen (endotoxin). We investigated the effect of hydrocortisone on hypothermia and survival time of adrenalectomized rats given a lethal dose

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of *E. coli* lipopolysaccharide (endotoxin)(7), kindly supplied to us by H. W. Schoenlein of Difco Labs. Since a large number of bioassays are available for glucocorticoids(2), it was visualized that development of an assay for hydrocortisone based on its protective effect against endotoxin in the adrenalectomized rat would provide basic information for development of an assay for corticotropin utilizing the hypophysectomized rat. Although corticosterone rather than hydrocortisone is the principal glucocorticoid elaborated by rat adrenal(8), Levitan, *et al.*(5) showed that corticosterone is effective in protecting the normal rat against a lethal dose of typhoid endotoxin. Using survival time as criterion of response, we determined dosage response curves for hydrocortisone in the adrenalectomized rat and corticotropin in the hypophysectomized rat to lethal doses of *E. coli* endotoxin.

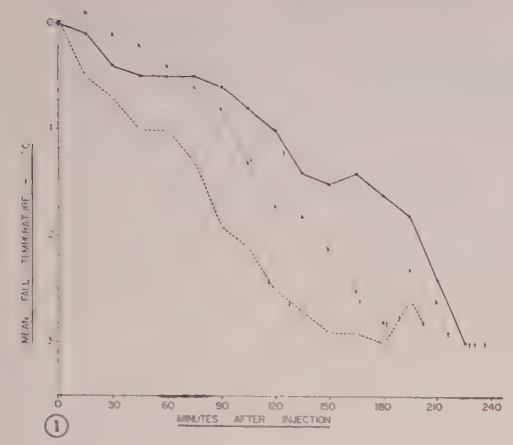
Materials and methods. Rats of Food and Drug Administrations stock colony derived from Osborne-Mendel strain were fed standard laboratory diet and water *ad lib.* All rats were adrenalectomized or hypophysectomized the day prior to use. Following death, all animals were examined grossly for completeness of extirpation of the glands. If glandular tissue was found, the animal was discarded. Suspensions of *E. coli* lipopolysaccharide in normal saline were prepared immediately before use. Endotoxin was administered intraperitoneally in 0.25 ml containing 0.5 mg. Hydrocortisone-free alcohol was dissolved in saline and administered intraperitoneally in 0.5 or 1.0 ml. Saline was administered to control animals. Solutions of U.S.P. Reference Standard Corticotropin were prepared in saline immediately prior to each experiment. Solutions were diluted so that dose was contained in 0.5 ml and administered intravenously. *Survival time* was recorded in minutes and measured from time of endotoxin injection until expiration of animal.

Results. The effect of hydrocortisone on body temperature and length of survival of adrenalectomized rats given endotoxin was studied. Male rats weighing approximately 300 g were adrenalectomized and the following day anesthetized with pentobarbital (30 mg/kg). Thermistors were inserted rectally and body temperatures recorded each 15 minutes. Animals were divided into 3 groups of 4 animals each and injected with 0.5 mg of *E. coli* endotoxin. Immediately thereafter one group received saline, one group 25 μg hydrocortisone, and the remaining group 50 μg hydrocortisone. The results are shown in Fig. 1. It will be seen that hydrocortisone decreased the fall in body temperature and prolonged the life of rats. Preliminary experiments had shown that this amount of endotoxin caused death of adrenalectomized rats in 1 to 3 hours, a factor of practical importance. Larger amounts of endotoxin offered no further advantage and smaller amounts resulted in greater variation in the results.

There was no difference in survival time of adrenalectomized rats given a lethal dose of endotoxin when total dose of endotoxin and hydrocortisone was given as a single intraperitoneal injection or divided into 2 equal injections 30 minutes apart.

To determine the best time to inject hydrocortisone to obtain greatest protection against the lethal effect of endotoxin, hydrocortisone was administered 15 or 30 minutes before endotoxin, simultaneously with endotoxin, 15 or 30 minutes following endotoxin, and in a divided dose, one-third of dose 30 minutes before, one-third simultaneously with, and one-third 30 minutes following the endotoxin. Maximum survival time was obtained when hydrocortisone was administered simultaneously with the endotoxin or as a divided dose. Hydrocortisone given 15 or 30 minutes before or 15 or 30 minutes following the endotoxin did not increase survival time.

No statistically significant difference was noted in response to hydrocortisone between young male and female adrenalectomized rats given a lethal dose of endotoxin. However, a statistically significant difference in survival times of young and old rats was found. Young male adrenalectomized rats weighing 64 to 186 g given 0.5 mg/100 g of body weight of endotoxin survived 115.8 $\pm$ s.e. 5.6 min. while older rats weighing 224 to 453 g survived 143.6 $\pm$ s.e. 5.0 min. There was no differ-



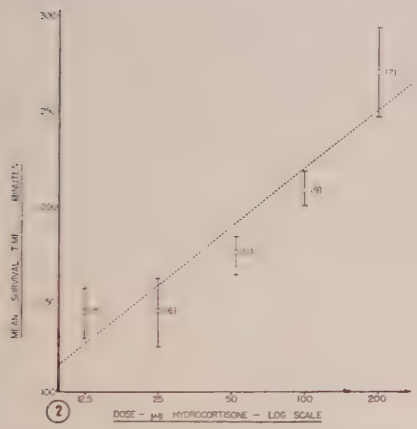


FIG. 1. Mean fall in rectal temperature and deaths (indicated by †) of male adrenalectomized rats inj. with 0.5 mg endotoxin followed immediately with saline (-○- - - -○-), 25 µg hydrocortisone (•×• • •×•), and 50 µg hydrocortisone (-●——●-). The animals were anesthetized with 30 mg/kg of pentobarbital.

FIG. 2. Dosage-response curve for hydrocortisone in male adrenalectomized rats weighing between 150 and 220 g given 0.5 mg endotoxin immediately followed by hydrocortisone in n-saline. Figures in parentheses indicate No. of animals at each dose level and vertical lines show stand. error of mean. Equation for the line shown was calculated from all dosage levels and is $y = 9.53 + 104.83x$, $\lambda = 0.373$. For doses 12.5, 25, and 50, $y = 78.83 + 55.83x$, $\lambda = 0.390$; for doses 25, 50, and 100, $y = 5.23 + 106.97x$, $\lambda = 0.278$; for doses 50, 100, and 200, $y = -93.90 + 156.38x$, $\lambda = 0.253$. Mean survival time of 7 rats given only endotoxin and n-saline was 124.7 ± 18.0 min.

ence in response to 50 µg of hydrocortisone, survival time being increased approximately 60 minutes in both age groups.

The dose response for hydrocortisone in young adrenalectomized rat given a lethal dose of endotoxin is shown in Fig. 2. Because of limited solubility of hydrocortisone in saline, doses higher than 200 µg were not used. The curve is for all doses except the zero dose, plotting survival time in minutes as the response *vs.* log of dose of hydrocortisone in µg. Analysis of variance showed that the line was linear. It will be seen that the index of precision as indicated by λ was considerably improved by selecting higher doses. Application of Bartlett's test(9) showed that variance between responses of groups was homogeneous.

A similar dose-response relationship was determined for the hypophysectomized rat given a lethal dose of endotoxin and various doses of corticotropin. The results of 5 such experiments are shown in Table I. None of the individual lines had a statistically significant slope. However, when all the results were combined in a single assay the analysis of variance showed that the slope was significant and that the relationship was linear when the log of dose was plotted *vs.* the response (equation for line, $y = 103.90 + 37.13x$). The index of precision, λ, for the combined results was 1.069.

From the above results it appears that an assay method based on protective effect of adrenal cortical steroids to endotoxin would have an index of precision and a sensitivity comparable with other methods of assay based on stress(2). For assay of corticotropin using the hypophysectomized rat, the method appears to be less satisfactory. It is possible that in routine use of the method, a better index of precision may be obtained. We have made no studies with regard to specificity of the method.

Summary. 1. Small doses of hydrocortisone decrease the amount of hypothermia and increase survival time of adrenalectomized rats given a lethal amount of *E. coli* lipopolysaccharide (endotoxin). 2. Using survival time as the criterion of response, the dose response relationships for hydrocortisone in the adrenalectomized rat and for corticotropin in the hypophysectomized rat were determined. When the log of dose was plotted *vs.* response, the lines were linear in ranges of 10 to 200 µg of hydrocortisone and 0.5 to 4.5 milliunits of corticotropin. The indices of precision were

TABLE I. Survival Time Response of Hypophysectomized Rats Weighing 80 to 150 g Given 0.5 mg of Endotoxin Intraperitoneally Followed Immediately by Intravenous Injection of Corticotropin (ACTH).

		Total dose of corticotropin in milliunits							
		0.0		0.5		1.5		4.5	
Day	Sex	No. rats	Mean survival time, min.	No. rats	Mean survival time, min.	No. rats	Mean survival time, min.	No. rats	Mean survival time, min.
1	♂	6	92.7 ± 14.9*	5	113.8 ± 19.5*	11	145.7 ± 8.2*	9	160.8 ± 12.1*
2	♀	9	136.7 ± 10.8	7	122.7 ± 10.7	11	148.8 ± 9.1	10	140.8 ± 6.4
3	♂	9	114.4 ± 9.4	6	130.7 ± 17.5	7	105.4 ± 10.5	7	196.7 ± 31.5
4	♀	9	123.2 ± 7.7	9	157.0 ± 11.2	9	163.8 ± 11.2	11	168.7 ± 10.8
5	♂	7	125.6 ± 9.0	5	113.0 ± 10.9	6	161.8 ± 22.4	8	173.2 ± 19.1
Combined		40	120.1 ± 8.4	32	130.0 ± 6.6	44	146.0 ± 5.6	45	166.1 ± 7.2

* Stand. error of mean.

approximately 0.3 and 1.0 for hydrocortisone and corticotropin, respectively. 3. The effect of sex and age of animals and time of administration on response to hydrocortisone were studied.

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Urinary Excretion of Beta Aminoisobutyric Acid (BAIBA) in Irradiated Human Beings.* (24548)

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Recently a previously unknown amino acid, beta-aminoisobutyric acid (BAIBA) has been identified in human urines by Crumpler, Dent *et al.*(1), and Fink *et al.*(2). Further studies by Fink using C^{14} labeled thymine indicate that BAIBA is a product of thymine metabolism(3,4) (Fig. 1). Since thymine is a pyrimidine peculiar to deoxyribonucleic acid (DNA), it appears likely that under certain circumstances BAIBA excretion may reflect alterations in DNA metabolism. Aminoaciduria has been reported following total body irradiation(5,6). Analysis of the amino acids studied indicated that glycine and taurine were excreted in increased amounts from presumed protein catabolism. Pyrimidine metabolites such as BAIBA have not been studied previously to our knowledge following total body irradiation, however, Awapara(7) noted increased BAIBA excretion followed nitrogen mustard or local x-ray treatment in some leukemic patients. In June 1958, in the course of an industrial operation involving uranium, a critical geometry accidentally occurred. Eight individuals nearby were exposed to the resultant mixed neutron and

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FIG. 1. Thymine metabolism.

gamma radiations. Five of the 8 persons were heavily exposed and developed early symptomatology. The remaining 3 with less exposure had no symptoms. A full description of the incident and the clinical courses of the involved personnel has been reported by Brucer and Andrews(8). The present studies concern measurements of these irradiated subjects, in whom large masses of radiosensitive tissues were undergoing dissolution and alteration.

Materials and methods. Frozen 24 hour urine aliquots were thawed and processed for BAIBA measurement using a modification of the method described by Awapara and Sato (9). Ten ml aliquots of the urines were mixed with 1 g portions Darco G-60 charcoal and heated briefly in a water bath. Following centrifugation 6.0 ml of the charcoal treated urines were placed on Dowex-2 columns (previously converted to the hydroxide form) for desalting. After the urinary supernatants had entered the resin beds, the columns were washed with cold boiled distilled water until the effluent became neutral. The columns were then eluted with enough 4N acetic acid to collect 10 ml. Eluates were lyophilized and resultant dried materials dissolved in 1 ml portions of distilled water. These concentrates were frozen in preparation for chromatography. Two-dimensional chromatography was used for identifying BAIBA. Ten lambda (.01 ml) of the concentrates were spotted on 8 inch squares of Whatman No. 4 paper held in the frame described by Datta *et al.*(10). Solvent 1 was the organic phase of n-butanol, acetic acid and water (5:1:4) and solvent 2 consisted of 2,4, lutidine saturated with water. After drying, the papers were sprayed with ninhydrin reagent and the color developed by heating at 100°C. Known amounts of BAIBA of varying concentrations were dissolved in water and chromatographed simultaneously. BAIBA was readily identified by Rf measurement (Rf 1 = 0.45 ± .02; Rf 2 = 0.32 ± .02). Color intensity and size of the ninhydrin spots were graded 0 to 5+ by 2 independent observers. Semiquantitative estimation of BAIBA in urines in μM/L were performed by comparison with standards and calculated on the basis of 24 hour urine volumes. Duplicate determinations were reproducible. Identical amounts of BAIBA have been added to BAIBA "negative" urine and also to equal volumes of water and then processed as above. Analyses of the urine samples always showed lower BAIBA content than BAIBA in water samples. The reasons for this are under study. The BAIBA detected in the experimental urines thus does not represent all of the BAIBA actually excreted. However the losses we believe are uniform(7).

Results. Table I shows the measured daily urinary BAIBA levels of 8 individuals hospitalized after radiation exposure. BAIBA is expressed in μM/Liter of urine. The 24 hour urine volumes are also recorded. The first urine samples for these studies were obtained on third day after exposure. BAIBA excretion was ranked according to quantity excreted, then the exposure doses supplied by Brucer and Andrews(8) were compared to this order. The fair parallelism of excretion with dose is evident. In Fig. 2 is shown the total μM BAIBA excreted per day in these patients.

Discussion. The data presented demonstrate that there is an apparent increase considerably above normal in BAIBA excretion in irradiated human beings. There are good indications that the excretion pattern is dose dependent. In our laboratory, maximum excretion in urines of normal individuals and in urines of patients with non-neoplastic disease has been between 0 to 150 μM/Liter of urine. Awapara(7) states that BAIBA excretion in normal individuals is an uncommon

TABLE I. Urinary BAIBA Excretion in 8 Patients following Irradiation.

Patient	1	4	3	5	2	6	7	8
Estimated exposure in rads(7)								
Neutrons	66	57	55	45	39	14	8	4
Gamma	210	182	175	143	114	43	34	17
Total	276	239	230	188	163	57	42	21
	BAIBA excretion, μM/l, and 24 hr urine vol							
Day 3	3+ 920	3+ 1975	2+ 3390	0 3150	+ 2320	2+ 1410	0 1220	0 2040
4	4+ 1300	3+ 1035	3+ 1610	2+ 1980	2+ 2290	0 1360	0 2940	0 3480
5	3+ 1700	2+ 1840	2+ 2015	+ 2250	+ 2420	*	*	*
6	3+ 1980	" 1970	" 1270	+ 1360	0 2815			
7	2+ 1850	" 1630	" 1780	+ 3080	+ 2575			
8	" 2680	+ 1870	" 2600	+ 3420	+ 2560			

0 = <75 μM/l BAIBA approximately.
\+ = 75 *idem*
2+ = 125 "
3+ = 250 "
4+ = 500 "

* No further specimen received.

event. These observations are consistent with more quantitative observation on normals by Fink *et al.*(11). There has been some variation in technics. However, we are quite confident that the amounts excreted by the irradiated human beings are significantly elevated, in spite of the semiquantitative nature of the method. Current studies on irradiated dogs showed a great increase in animals receiving 400 r whole-body irradiation appearing within 18 hours after exposure. BAIBA was not detected pre-irradiation and in normal dog urines. Investigation of the suggested dose dependency is underway in dogs.

The mechanism of production of increased BAIBA excretion is not clear. Studies by Caravaca and Grisolia(12) suggest that BAIBA may arise in part directly from sources other than thymine such as carbamyl amino acids. However, the significance of this pathway in mammals needs further evaluation. The pattern of excretion seen here follows the extensive necrosis of radio-sensitive cells of lymph nodes, bone marrow and GI tract. DNA destruction is manifested by pyknosis, phagocytosis and disappearance of large amounts of Feulgen staining material. The pathway of destruction of DNA after radiation injury is not known. It is however a potential source of urinary BAIBA after irradiation. It has been shown that intravenous H^3 thymidine in human beings and other animals is either incorporated into DNA or catabolized(14). Therefore in respect to this pyrimidine deoxyriboside there are at least 2 pathways by which it is utilized. It is believed that some of the thymidine not incorporated into DNA is promptly degraded through a series of steps to BAIBA.† Accordingly when total DNA synthesis is decreased from any cause, metabolism of thymine compounds may be shunted towards BAIBA production.

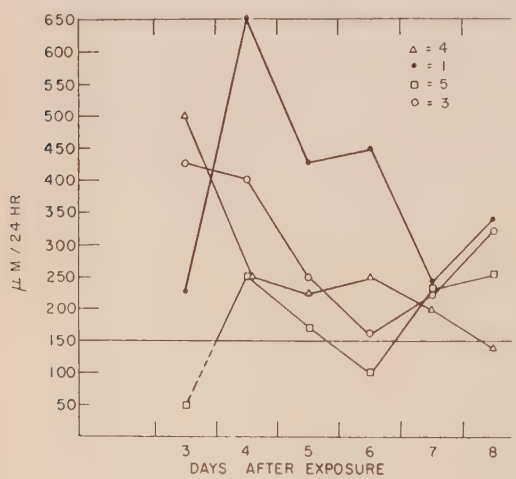

FIG. 2. Daily urinary BAIBA excretion after irradiation.

These alternatives are subject to experimental attack. If the BAIBA comes from destruction of cells by irradiation and if DNA

† Chromatography of urine from one of our patients who received intravenous H^3 thymidine was done by Dr. George Gerber at the University of Rochester, School of Medicine and Dentistry, Div. of Exp. Radiol. He has isolated and characterized tritium containing BAIBA from the urine.

in cells were labeled before irradiation with H^3 thymidine, some of the BAIBA excreted after irradiation should have a tritium label if DNA breakdown contributes BAIBA. Increases of urinary BAIBA detected following irradiation may prove useful in early detection of serious exposure.

Summary. 1. Beta-aminoisobutyric acid (BAIBA) has been found in increased amounts in urine from a group of human beings accidentally exposed to serious radiation. 2. BAIBA levels excreted appear related to the estimated doses received. 3. The implications of BAIBA excretion as related to DNA metabolism and irradiation-induced aplasia are discussed.

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Anticonvulsant Effect of Dilantin Sodium by Intravenous Administration in Mice. (24549)

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Dilantin sodium®* given intravenously has, in recent years, been advocated for treatment of status epilepticus and prevention of seizures in neurosurgery(1,2). The principal advantage of Dilantin, as contrasted to hypnotic and anesthetic agents, is high anticonvulsant potency unaccompanied by depressant effects on the respiratory mechanisms. Because the recommended dose of 250 mg of intravenous Dilantin sodium was found in some cases incapable of promptly suppressing epileptic seizures, the question has been raised as to whether this lack of prompt anticonvulsant effect could be a result of insufficiency in dosage or might be caused by some factor which delays its action on the central nervous system (personal communication). Accordingly, rate of onset and duration of anticonvulsant effect of Dilantin on electrically-induced seizures were investigated in mice at various dosages following intravenous administration.

Methods. The drug (Steri-Vial Dilantin sodium), dissolved in water, was injected *via* the marginal tail vein of male albino mice weighing 18 to 22 g. Electroshock as described by Toman, Swinyard and Goodman (3) with slight modification(4) was employed to produce convulsive seizures. The anticonvulsant effect of Dilantin was determined either (a) by finding a dose of the drug required to abolish the tonic-extensor seizures in 50% of the mice (PD_{50}) when a fixed current strength was chosen to induce convul-

* Dilantin® is trademark applied to dihpenylhydantoin by Parke, Davis & Co.

TABLE I. Anticonvulsant Effect of Dilantin Sodium by Intravenous Administration in Mice.

Current, ma	Time after dosing			
	1 min.	5 min.	15 min.	60 min.
	*PD_{50} ± S.E., mg/kg intrav.			
6	13.6 ± .8	9.9 ±.6	7.8 ±.3	8.0 ±.3
9	19.6 ±1.1	15.5 ±.5	11.0 ±.2	10.4 ±.3
12	25.5 ±1.0	14.2 ±.4	14.6 ±.2	10.9 ±.3
24	25.5 ± .4	20.5 ±.7	15.5 ±.1	12.0 ±.3
48	28.0 ± .7	21.5 ±.9	17.3 ±.2	14.8 ±.4

* 50% protective dose and stand. errors.

sions, or (b) by ascertaining the current strength necessary to produce tonic-extensor seizures in 50% of the animals (CS_{50}) when a protective dose of Dilantin was given. Three groups of 10 mice each were used for the 3 doses of Dilantin (for a fixed current strength) or for the 3 current strengths (for a protective dose of Dilantin) that would give tonic-extensor convulsions in 10 to 90% of animals. PD_{50} or CS_{50} and standard errors were estimated graphically by the method of Miller and Tainter(5).

Results. Table I gives the results obtained from experiments in which convulsions in mice were produced by a fixed current strength at 6, 9, 12, 24 or 48 milliamperes. The anticonvulsant effect of Dilantin was determined at 1, 5, 15 and 60 minutes after intravenous injection. The data indicate first, as is to be expected, that the amount of Dilantin (PD_{50}) required for abolishing the tonic-extensor seizures was proportionally greater for convulsions induced by higher current strength. A significant feature is that this was also found to be the case for Dilantin one minute following intravenous administration. Second, as revealed by the lower protective doses of Dilantin from 1 to 60 minutes after injection for convulsions which were induced by the same current strengths, there was an increasing anticonvulsant effect of Dilantin during this period.

Rate of onset and duration of anticonvulsant effect of Dilantin are more clearly shown by the graphic data in Fig. 1, which was constructed with the results of another experiment by plotting current strength required to produce tonic-extensor seizures in 50% of mice (CS_{50}) against time interval after the animals had received 15 mg/kg of Dilantin sodium intravenously. It is seen that the anticonvulsant effect of Dilantin (in terms of the CS_{50} value) became increasingly greater immediately following injection, reached a peak at about 30 minutes and peak value was maintained for the next 1½ hours. A significant anticonvulsant effect was evident 8 hours after administration. Similarly, the effect of 5 mg/kg of Dilantin sodium was also investigated. This is the dosage recommended for management of epilepsy in humans. The control CS_{50} was 5.00 ± 0.10 and significant increases were shown at 15 minutes, CS_{50} = 5.30 ± .09 (P = .01) and at 30 minutes CS_{50} = 5.52 ± .12 (P = .01). No significant differences were obtained at 1 minute, 5 minutes and 1, 2, 4 and 6 hours. Apparently this is the minimal dose of Dilantin sodium required to show an anticonvulsant effect on electrically-induced seizures in mice.

Discussion. In view of the fact that Dilantin is effective in abolishing tonic-seizures in mice one minute after intravenous administration, it appears that Dilantin reaches the site of action in the central nervous system and reacts with the receptors very quickly. Since the tonic-extensor seizures induced in mice at higher current strength can be abolished by Dilantin with a corresponding increase in dosage, those cases of status epilepticus which were not promptly controlled by 250 mg of intravenous Dilantin sodium, could very likely be relieved by higher doses of the drug.

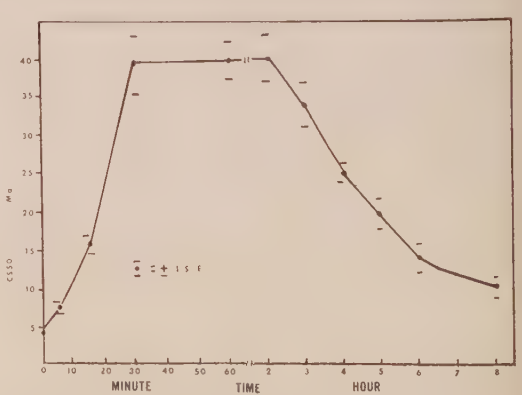

FIG. 1. Rate of onset and duration of anticonvulsant effect of a fixed dose of Dilantin sodium (15 mg/kg) following intrav. administration in mice.

The prolonged anticonvulsant effect of Dilantin shown by intravenous administration in mice, if true also in man, is certainly a very desirable property of the drug in management of status epilepticus or for prophylactic control of seizures in neurosurgery.

Summary. 1. Rate of onset and duration of anticonvulsant effect of intravenous Dilantin sodium against electrically-induced tonic-extensor seizures have been investigated in mice. At 15 mg/kg, the anti-convulsant effect of Dilantin reached a peak approximately 30 minutes after injection, and was maintained for 1½ hours; it then began to decline gradually, persisting more than 8 hours. 2. Dilantin, one minute after intravenous injection, was capable of abolishing the tonic-extensor seizures in mice at doses in proportion to the current strengths employed to produce convulsions.

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Canine Hepatitis Virus: Attempts to Find Relationship with Human Hepatitis.*† (24550)

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Two groups of investigators(2,8) described successful isolation and propagation of canine hepatitis virus in cell cultures of dog kidney. Since then the growth of this virus in cell cultures of kidney tissue from other animals has been reported(5,9). Fastier(6,7) described agglutination of fowl erythrocytes by this virus using infected tissue culture fluids as antigens. This agglutination was inhibited by immune sera from dogs naturally or experimentally infected with canine hepatitis virus. Although there is no epidemiologic evidence to suggest that the infectious agent of human hepatitis is the same as that of canine hepatitis, the possibility of some immunologic relationship between the two was felt to warrant exploratory investigation using recently developed methods.

Materials and methods. Virus. A strain of canine hepatitis virus, which had been passed 37 times in dog kidney tissue cultures, was kindly supplied by Dr. V. J. Cabasso, Lederle Medical Research Dept. *Cell cultures.* Kidneys from young puppies were trypsinized according to the method of Rappaport(16). Cultures were prepared by seeding each tube with about 300,000 cells contained in 1 ml growth medium. In the same way cultures were prepared from rhesus monkey and young pig kidneys. The growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (BSS)(13). For dog and pig kidney cell cultures this was supplemented with 10% calf serum; for rhesus kidney cell cultures with 2% calf serum. From continuous cell lines§ of human origin: Detroit-6(1);

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§ Original cell lines were kindly supplied by: Drs. I. W. McLean and W. A. Rightsel, Parke Davis and Co., Detroit, Mich., (Detroit-6 cells); Dr. Alice E. Moore, Sloan-Kettering Inst., N. Y., (Hep-2 cells); Dr. Jørgen Fogh, Virus Laboratory, Berkeley, Calif., (FL cells).

FL(10); and Hep-2(14), cultures were prepared by seeding each tube with approximately 100,000 cells contained in 1 ml of growth medium consisting of Eagle's medium (4) with 10% calf serum. Prior to inoculation of virus the Eagle's medium was replaced by 2 ml of medium 199(15). *Neutralization tests.* Serial dilutions of sera were made in medium 199. Each dilution was mixed with equal quantity of virus *i.e.* 0.2 ml of serum-virus mixture contained about 100 TCD_{50}. After one hour at 37°C, 0.2 ml of each mixture was inoculated into 2 or 3 tube cultures of dog kidney epithelium. Final readings were made at 5 to 7 days when control titration showed the desired amount of virus to be present. CF tests were carried out according to the method of Fulton and Dumbell(11) as modified by Svedmyr, Enders and Holloway (18). High titer tissue culture fluids were used as antigens. All sera were inactivated at 56°C for a half hour. Sera to be used as antigens in HA test were extracted with acetone and ether and reconstituted in saline according to method used by Havens for sera from human hepatitis patients(12). The HA reaction was carried out according to methods described by Clarke and Casals(3). Serial dilutions of antigens, either reconstituted serum samples or tissue culture suspensions of canine hepatitis virus were made in borate saline buffer (pH 9.0). To 0.5 ml of each dilution was added 0.5 ml of a 0.25% suspension of one-day-old-chick erythrocytes in various pH adjusting diluents consisting of phosphate buffers. These solutions were used to adjust the pH in final HA reaction to any desired pH between 6.0 and 7.6, when mixed with equal amounts of antigen diluted in borate saline.

Results. Canine hepatitis virus was routinely propagated in dog kidney monolayer cell cultures. Previously described(2,8) cytopathic effects (CPE) were observed. That this virus can be cultivated in porcine kidney cell cultures(5,9) was confirmed. Rhesus monkey kidney cultures, Detroit-6, FL, and Hep-2 cell cultures inoculated with dog kidney passage virus produced changes in each after 3-4 days. At various time intervals subcultures were made in cells of the same type. In no case, however, did CPE develop in series of 3-4 blind passages.

Two puppies were injected with canine hepatitis virus passed in dog kidney cell culture. Both survived the mild disease which resulted. Preinoculation serum samples contained no neutralizing, CF, or hemagglutination-inhibiting antibodies. Sera obtained 4 weeks after inoculation contained neutralizing antibodies in titer greater than $1 = 160$ and both inhibited 8 HA units at dilution of 1:40. No CF antibodies were demonstrable. The dogs were bled 5 times during first week after fever. We used these sera, after extraction with acetone and ether, as HA antigens in a reaction with one-day-old-chick erythrocytes. No HA was observed at pH values of 6, 7, and 7.6.

Neutralization experiments were carried out with various human sera against canine hepatitis virus. Paired sera from 7 (including 1 case of serum hepatitis) and single convalescent serum samples from 5 hepatitis patients were tested. As controls, serum samples were included from 4 normal persons. None of these sera gave neutralization in dilutions 1:5 and 1:10. In all tests sera from immune dogs were included as positive controls. These sera were also tested by CF for presence of antibodies against canine hepatitis virus. The antigen, consisting of undiluted culture fluid, had a titer of 4. As control an immune dog serum (titer 1:32) was used. All tests with human sera gave negative results at lowest dilution tested (1:8).

By using the above HA technic, maximal HA titers for canine hepatitis virus tissue culture antigens were at pH 6.0. At higher pH values tested, lower titers were obtained. This agglutination was not inhibited by normal human sera, 4 acute phase and 10 convalescent phase sera from human hepatitis patients. In each test, serum from an immune dog was included as control.

Discussion. Our purpose was to investigate the possibility of a serologic relationship between canine hepatitis virus and the infectious agent of human hepatitis. Such a relationship could not be demonstrated by the indirect method of testing acute and convalescent sera from human hepatitis patients

against canine hepatitis virus in complement fixation, neutralization or hemagglutination inhibiton tests. This indicates that there is no antigenic relationship between human and canine agents. Even though only one strain of canine hepatitis virus was included in this study, it is unlikely that the results might be altered by using other strains since by means of complement fixation and cross neutralization tests performed in dog kidney cell cultures Salenstedt(17) was unable to demonstrate any significant antigenic difference between 5 strains of canine hepatitis virus.

Fastier's findings(6) that canine hepatitis virus agglutinates chick erythrocytes and that this agglutination could be inhibited by specific immune serum were confirmed. In his system maximal titers were at pH 7.5 to 8.0. In the HA reaction employed by us, maximal titers occurred at pH 6.0. This may be accounted for by differences in the buffers used.

Havens(12) has described that acetone-ether extracted sera from human hepatitis patients may contain agglutinins for chick erythrocytes. In our study this method was applied to serial acute phase sera from 2 dogs inoculated with hepatitis. No hemagglutinins were demonstrated. It may be that this phenomenon does not occur in canine hepatitis, or that the disease in the 2 dogs was too mild.

By inoculaion of canine hepatitis virus into cultures of rhesus monkey kidney cells and cultures of various human cell lines characteristic cytopathic changes resembling those observed in dog kidney cell cultures, developed in the first passage, but not subsequently.

Summary. 1) Observations on growth and behavior of a strain of canine hepatitis virus in various tissue culture systems of human and animal origins have been described. 2) By testing acute and convalescent phase sera from human hepatitis patients against canine hepatitis virus in neutralization, complement fixation, and hemagglutination inhibitions tests, no antigenic relationship could be demonstrated between this virus and the infectious agent of human hepatitis.

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Concentration of Corticotropin Releasing Factor by Chromatography on Carboxymethylcellulose. (24551)

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Evidence has been obtained that minute doses of a corticotropin releasing factor (CRF) purified by paper chromatography and electrophoresis are active in stimulating release of corticotropin from the anterior pituitary tissue *in vitro*(1,2). The effect of these preparations to increase incorporation of P^{32} into the pituitary phospholipids, interpreted as an indication of active secretory process has also been described(3). Though it was possible to obtain amino acid composition of purified material(2) amounts obtained were not sufficient for homogeneity, degradation and structural studies. This communication describes large scale purification of CRF on carboxymethylcellulose (CMC) cation exchange adsorbent on the basis of an *in vivo* assay for CRF.

Methods and materials. CMC was prepared as described in Schally *et al.*(4) except for additional treatment with 8-hydroxyquinoline after synthesis. The operation of columns and other details of chromatography have also been described(4). Starting materials for chromatography were protopituitrin and pitressin intermediate (Parke, Davis and Co.) of posterior pituitary origin, which are fractions C and D in the Kamm procedure as outlined by Waring and Landgrebe(5).* Protopituitrin of hog origin was treated 3 X with oxycellulose to remove ACTH(6). Elution of peptide and protein material was effected by use of ammonium acetate buffers of progressively increasing ionic strength and pH (4). Bracketed assays for oxytocin were performed by vasodepressor assay of Coon(7) as modified by Thompson(8) and pressor activity was measured in the rat(9) against USP XV posterior pituitary reference standard. CRF activity as evidenced by stimulation of ACTH release was assessed by measurement of plasma free corticosteroids in the rat(10) after morphine-nembutal administration. All fractions were tested at a dose corresponding to 10 or 30 milliunits pressor activity as determined previously by assay for vasopressin (see above). This low dose of vasopressor activity, as highly purified lysine vasopressin (287 U/mg, obtained by separation on CMC(11) followed by paper partition chromatography in m-cresol/H_2O(2)) is devoid of hypophysiotropic activity in the nembutal-morphine preparation(12,13). The results are expressed in provisional CRF units using crude CRF preparation fraction D(1) as standard. Response of the nembutal-morphine blocked rat to fraction D is similar to that of the rat 20-24 hours after effective hypothalamic lesion(12). All fractions showing CRF activity are finally tested in the 24 hour hypophysectomized rat.

Results. Figs. 1 and 2 show the chromatographic pattern and biological activities obtained. It is apparent from Fig. 1 that pressor and CRF activities were closely related in their chromatographic behavior on the small column of carboxymethylcellulose. The maximal CRF activity, however, was found in the early tubes of the vasopressin peak. Fraction No. 610 was active to release ACTH at level of 0.06 μg and was 4-5 times as active as fraction No. 630, though both these fractions had the same pressor activity of 260 U/mg. Fraction No. 590, was more potent CRF-wise than fraction No. 630, though pressor activity of No. 590 was only half that of No. 630.

Fig. 2 shows that with a large column and different gradients a better separation of the 2 activities was obtained, even though specific activity of CRF fractions was lower in the second column than in the first, due to presence of large amounts of other contaminating peptides or proteins but different from vasopressin and oxytocin. Fractions No. 640-680 had most of the CRF activity and only 13-25

* Evidence has been reported that CRF is present in both hypothalamus tissue and commercial extracts of posterior pituitary(1,2).

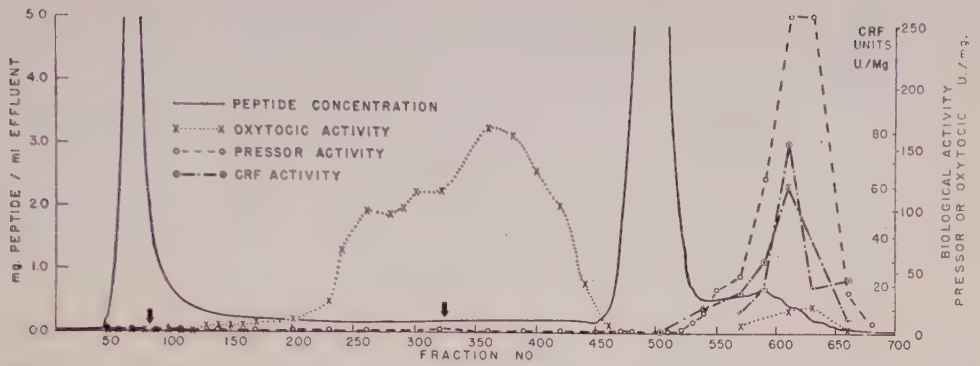

FIG. 1. 1 g protopituitrin applied on column 1.5 × 47 cm, equilibrated with 0.01 M, pH 4.5 ammonium acetate buffer. Hold-up vol 50 ml. Gradient to pH 6, 0.02 M buffer started at tube 85 through a 125 ml flask. Gradient to 0.2 M, pH 7 buffer started at tube 323 through a 250 ml flask. 1.2 ml fractions collected.

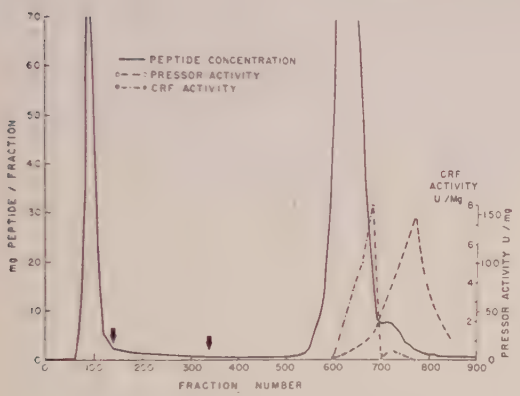

FIG. 2. 10 g pitressin intermediate applied on column 3.8 × 52 cm. Hold-up vol 300 ml. Gradient was 0.01 M, pH 4.5 to 0.02 M, pH 6 buffer through 500 ml flask. Second gradient was to 0.2 M, pH 7 buffer through 2000 ml flask started at tube 340. 5 ml fractions collected.

U/mg pressor activity. Fractions 700-770 had no CRF activity at a dose of 30 pressor milliunits.

The fractions containing CRF were tested in the 24 hour hypophysectomized rat for ACTH at doses twice those injected for the CRF assay. All were found to be inactive in the hypophysectomized rat.

Summary. 1) Neurohypophysial extracts of commercial origin were chromatographed on carboxymethylcellulose cation exchange adsorbent under conditions of pH and ionic strength gradient in view of separating CRF. An *in vivo* assay was used to assess CRF activity. 2) The method described is useful in large scale preparations. Fractions containing CRF were regularly found to emerge before lysine vasopressin and were well separated from oxytocin.

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Absorption of H^3-β-Sitosterol in the Lymph Fistula Rat.* (24552)

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It has been established that plant sterols inhibit absorption of cholesterol and presumably, by this mechanism, they thereby lower blood and tissue levels of cholesterol(1-3). Furthermore, this inhibiting effect of plant sterols may be due to competition with cholesterol for the sterol absorptive system in the intestinal wall(4,5). After administration of H^3-β-sitosterol there was increased fecal excretion of cholesterol and related sterols(5). It was also observed that a considerable portion of H^3-β-sitosterol was not recovered in the feces and was presumably absorbed or converted to substances not precipitable with digitonin. Gould(6) fed tritium labeled sitostrol to humans and animals and from balance and blood data concluded that it was absorbed to a slight extent. Sitosterol balance studies in humans and rats have indicated considerable absorption of up to 40%(7-9). However, such experiments do not supply data on mechanism or route of absorption. Recent reports(10,11) on the mechanism of cholesterol absorption have indicated that there exists in the intestinal wall a pool with which cholesterol entering from the lumen is mixed prior to its esterification and transfer to the lymph. In light of these findings and the lack of definitive data on the mechanism of sitosterol absorption, it became of interest to determine the extent to which sitosterol enters the intestinal wall and is transferred to the lymph.

Methods and materials. Preparation and care of the thoracic duct fistula rats has been described(10). Twenty-four hours after operation each experimental animal received, by intubation, without anesthesia, 3 ml of aqueous emulsion containing 44 mg H^3-β-sitosterol† (6.8 μc), 50 mg blood albumin, 292 mg oleic acid, 279 mg sodium taurocholate, and 150 mg glucose; control rats received a comparable emulsion without sterol. There were 3 experimental groups of animals; A was killed at 6, B at 24, and C at 48 hours after the test meal. Lymph was collected in consecutive periods from 0-6, 6-24, 24-48 hours to time of sacrifice for each group. When animals were sacrificed, the small intestine was removed and saline washings from intestine were pooled with feces of the same animal. This gave combined samples of feces and intestinal contents for 0-6, 0-24, and 0-48 hours for groups A, B, and C, respectively. Lipid extracts of small intestine or segments of the intestine, and a non-saponifiable lipid extract of combined feces and intestinal contents were prepared according to procedures described earlier(10,11). Free and total cholesterol were determined colorimetrically on each extract of lymph and intestine; gravimetric sterol determinations were carried out on extracts of the combined feces and intestinal contents(10). Tritium activity of free, esterified, and total sterol fractions of all extracts were determined as previously described(5).

Results. At the end of 6-hour absorption period 8.7 mg or 19.9% of the fed H^3-β-sitosterol was not recovered in intestinal contents and feces (Table I). Analysis of feces and intestinal contents after 24 and 48 hour periods showed further disappearance of the fed sitosterol, 13.5 mg or 31.6% at 24 hours and 17.4 mg or 39.5% at 48 hours. Sterol absorption calculated from gravimetric sterol determination also indicated disappearance of the fed sitosterol, but such disappearance was less than indicated by activity data. These differences can be explained by the increase in fecal excretion of unlabeled cholesterol or related sterols when H^3-β-sitosterol is fed. Thus, for 24 hours excretion of unlabeled sterol increased from 6.0 to 15.9 mg. Also, specific activity of the sitosterol in the intestinal lumen indicated considerable dilution of the fed H^3-β-sitosterol with time.

* This study was supported by grants from Am. Heart Assn. and PHS.

† Kindly supplied by Eli Lilly Research Labs.

TABLE I. Recovery of Fed H^3-β-Sitosterol from Feces and Contents at Different Time Periods.

Time, hr	Feces and intestinal contents: Sterols total, mg	H^3-β-sitosterol, mg	Sterol other, mg	Specific activity† total sterol, μc/mg	H^3-β-sitosterol not recovered: mg	%
6	43.5 ± 2.2‡	35.7 ± 1.9	7.8 ± 1.4	.124 ± .011	8.7 ± 2.5	19.9 ± 5.9
24	46.4 ± .5	30.5 ± 3.5	15.9 ± 2.1	.100 ± .014	13.5 ± 2.6	31.6 ± 4.8
48	45.3 ± 2.5	26.6 ± 2.5	18.7 ± 1.3	.090 ± .010	17.4 ± 2.0	39.5 ± 3.8
24*	6.0 ± .4		6.0 ± .4			

* Represents 24 hr control excretion.
† Total H^3-β-sitosterol fed was 44 mg with specific activity of 0.154 μc/mg, 4 animals/group.
‡ Stand. error of mean.

At the end of 6 hours, 2 mg or 4.6% of the fed H^3-β-sitosterol was present in the intestinal wall (Table II). At 24 hours there was .8% and at 48 hours there was no H^3-β-sitosterol present. Virtually all of the H^3-β-sitosterol was present as free sterol; only a trace was present as esterified H^3-β-sitosterol. There was a small apparent increase in amount of free cholesterol in the intestinal wall at end of 6 hours. This increase could be accounted for by the uptake of H^3-β-sitosterol.

At various time intervals only traces of H^3-β-sitosterol could be detected in the lymph. These amounts were less than .3% of the fed dose. Also, there was no increase in the lymph total sterol level when compared to a group not receiving sitosterol in the test meal.

To locate area of maximum uptake of fed H^3-β-sitosterol by intestinal wall, the small intestine was sectioned into different length segments. The results were essentially the same as previously reported for cholesterol-4-C^{14} (11). About 70% of the H^3-β-sitosterol in the small intestine was present in the proximal half.

Discussion. Our results have confirmed and extended our earlier investigations (4,5,7). After feeding of H^3-β-sitosterol, there was increased fecal excretion of cholesterol and related sterols and from the balance data an apparent absorption of β-sitosterol. Based on these findings it was previously postulated that H^3-β-sitosterol was absorbed by the same mechanism as cholesterol and competed with cholesterol for the sterol absorptive system (5). The present results indicate that a considerable portion of ingested H^3-β-sitosterol is not accounted for by that in the lumen, intestinal wall, and lymph. The failure to account for all of the administered H^3-β-sitosterol in these 3 sites and the apparent absorption, from balance data, raises important questions regarding plant sterol absorption. These find-

TABLE II. Uptake of Fed H^3-β-Sitosterol by Intestinal Wall and Its Transfer to Lymph.

Time, hr*	No. rats†	Sterol free (mg)	H^3-sterol free (mg)	Sterol ester (mg)	H^3-sterol ester (mg)	Sterol total (mg)	H^3-sterol total (mg)	Admin. H^3-β-sitosterol recovered, %
				Small intestine				
6	4	15.1 ± 1.0§	2.01 ±.50	1.3 ±.2	tr	16.4 ± .9	2.01 ±.50	4.6 ± 1.1
24	4	12.9 ± 1.1	.33 ±.10	1.0 ±.2	.0	13.9 ± .9	.33 ±.10	.8 ± .2
48	4	13.6 ± .5	.0	.6 ±.1	.0	14.2 ± .6	.0	.0
				Lymph				
0– 6	12	1.2 ± .4	tr‖	1.6 ±.3	tr	2.8 ± .3	tr	<.3
6–24	8	3.4 ± .3	tr	4.7 ±.5	tr	8.1 ± .8	tr	"
24–48	4	3.6 ± .4	.0	6.2 ±.6	.0	9.8 ±1.0	.0	.0
0–24‡	5	3.3 ± .3		6.1 ±.3		9.4 ±1.1		

* Represents collection time of lymph and time of sacrifice after test meal.
† Total H^3-β-sitosterol fed was 44 mg with a specific activity of .154 μc/mg.
‡ Represents 24 hr lymph collection period of control group receiving sodium taurocholate and oleic acid.
§ Gravimetrically determined.
‖ tr = trace.

ings suggest that part of the fed H^3-β-sitosterol may have undergone transformation in the intestinal wall or lumen so that it was no longer precipitated with digitonin and thus, would not have been detected in these experiments. Such changes occur with fed cholesterol-4-C^{14}(11). Another less probable alternative is that H^3-β-sitosterol was absorbed by some other route than through the lymphatic system. We are now using C^{14}-β-sitosterol to test these possibilities.

The present data also show that a considerable amount of H^3-β-sitosterol, like cholesterol(11), is taken up by the intestinal wall and is present almost entirely as free sterol. However, the amount taken up was about one-half that of cholesterol. Roth and Favarger (12) have also observed uptake of D-sitosterol by the intestinal wall. Thus, in this first step of the absorption process both sterols appear to be handled in a similar fashion, albeit, β-sitosterol is taken up to a lesser degree. After free cholesterol is transferred into the intestinal wall it is largely esterified and then transferred into the lymph as part of the chylomicron(11). It appears that β-sitosterol is not esterified in the intestinal wall or transferred to lymph in appreciable quantities. It should be emphasized that if the amount of H^3-β-sitosterol present in intestinal wall at end of 6 hours, had been transferred to the lymph during the next 18 hours, it could have been easily determined by the technics employed.

Summary. Administration of 44 mg of H^3-β-sitosterol to lymph fistula rats and analysis of feces, intestinal contents, intestine, and lymph gave the following results: (1) balance data indicated approximately 40% absorption in 48 hours, (2) there was increased fecal excretion of cholesterol and related sterols, (3) there was considerable uptake of H^3-β-sitosterol by the intestinal wall, but essentially no transfer into the lymph. Explanations for the apparent disappearance of H^3-β-sitosterol are discussed.

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Proteolytic Enzyme System of Skin. VI. Enzyme Patterns in Various Animal Species.*† (24553)

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Several enzymes which comprise, in part, the proteolytic enzyme system of rat skin have been described by Martin and Axelrod (1-5). In extracts prepared from rat skin acetone powder, 4 distinct enzymes, designated Proteinase A, Proteinase C, the A_1-esterase, and the A_2-esterase, have been characterized, partially purified, and in some cases separated from each other. With the exception of Proteinase C, synthetic sub-

strates are known for these enzymes. Proteinase A, in addition to its ability to hydrolyze a protein substrate will also hydrolyze acylated and non-acylated aromatic amino acid esters such as N-acetyl-L-tyrosine ethyl ester (ATEE)‡ and TEE(2,4). The specificity of the A_1- and the A_2-esterase is not as inclusive, each possessing one facet of the substrate specificity of Proteinase A. Thus, the A_1-esterase will hydrolyze only *acylated* aromatic amino acid esters, *e.g.*, ATEE, and conversely, only *non-acylated* aromatic amino acid esters, *e.g.*, TEE and PEE, are hydrolyzed by the A_2-esterase. The hydrolysis of TEE and PEE by A_2-esterase occurs at equal rates(5). Despite overlapping specificity patterns of Proteinase A, the A_1-esterase, and the A_2-esterase, the activity of each can be determined by a differential assay procedure using ATEE, TEE, and an inhibitor to Proteinase A (4,5). The Proteinase A inhibitor, AIn (isolated from sheep serum), is specific for Proteinase A in the sense that it does not inhibit activity of any of the other above mentioned enzymes. The distinguishing characteristic of Proteinase C in an initial skin extract is its ability to undergo reversible inactivation; a time-dependent process proceeding optimally in solutions of ionic strength 0.6 to 0.8(1). The reverse or activation reaction is essentially instantaneous upon exposure of the inactivated extract to ionic strength 1.4 or greater (1). This behavior is due to the ionic strength-dependent association and dissociation of Proteinase C with a skin inhibitor. Both components have been separated from each other and studied in some detail(3). Thus, with casein as test substrate, that portion of total proteolytic activity that can be *reversibly* inactivated by suitable changes in ionic strength can be ascribed to Proteinase C. Since we are interested in ascertaining the importance of skin proteolytic enzymes in the cellular disruptive processes occurring as sequelae to either a thermal insult or an antigen-antibody reaction and since use of animals other than rat can be envisioned in such studies, it was considered of importance to obtain information on the ubiquity of rat skin-type proteolytic enzymes in the skins of other animals. The case for proteolytic enzyme activation by such means has received support by various investigators(6-14). In the present paper, extraction technics and methods of assay developed by experience with enzyme activities in rat skin acetone powder have been applied to fresh skin preparations of the rat and other animals. A brief report of some of this material has been presented(15).

Materials and methods. Preparation of extracts. Abdominal skin samples of various animals were freed of hair and underlying superficial fascia. Each skin was then scissor-minced and passed through a motorized Latapie Tissue Grinder§ in cold room to give a skin mince of approximately the same particle size for all skins used. After extraction of the mince for 18 hours at 1°C in 1.6 M KCl (4 ml/g), the suspension was centrifuged at 25,-

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‡ TEE, L-tyrosine ethyl ester; PEE, L-phenylalanine ethyl ester.

§ A Latapie Tissue Grinder, Arthur H. Thomas Co., Philadelphia, Pa., was modified and motorized as follows: a) the plunger crank was replaced with a 3 inch knurled wheel to give greater control over plunger displacement; b) the shaft attached to revolving plate was strengthened by increasing its outer diameter to approximately 5/16 inch; c) the mount was discarded and a new mount, incorporating a set screw partly into the chamber housing, was devised to prevent rotation about the long axis when the revolving plate shaft was attached to the motor; d) attachment to the motor was effected through a flexible coupling and both the motor and tissue grinder were rigidly mounted on an aluminum plate; and e) the series wound motor was obtained from Wilfred R. Uffelman, Bodine Electric Co., Cleveland, O. The torque rating was 19 inch-lb at 250 rpm. We found that even with this powerful motor, an appreciable slowing-down occurs upon passage of fresh scissor-minced skin through the grinder.

TABLE I. Esterase and Proteinase Activity of Skin Extracts.

Animal	mg protein per ml extract	$[k_0]^{ATEE}$		$[k_0]^{TEE}$	$[k_0]^{PEE*} / [k_0]^{TEE*}$	Proteinase activity ($\times 10^3$)	
		Total (I)	With AIn (II)	(III)	(IV)	Activation conditions (V)	Inactivation conditions (VI)
Rat	10.6 ± .6	1.71 ± .08	.83 ± .00	.32 ± .02	1.1 †	60 ± 10	23 ± 3
Guinea pig	7.3 ± 1.4	.45 ± .04	.46 ± .04	.18 ± .00	.67	9 ± 1	6 ± 1
Rabbit	8.9 ± 2.5	.0	.0	.28 ± .00	3.8	0	0
Hog	3.5 ± .8	.0	.0	.15 ± .05	2.7	0	0
Sheep	6.3 ± .8	.23 ± .05	.31 ± .04	.37 ± .08	1.9	0	0
Cow	1.9 ± .3	.0	.0	.0		0	0
Chicken	4.2 ± 1.2	.0	.0	.0		0	0
Monkey	4.7 ± .1	.35 ± .04	.35 ± .06	.11 ± .03	.68	18	11
						28	14
						41	12

* Activity determinations performed on pooled samples of skin extracts.
† Corrected for contribution of Proteinase A to hydrolysis of TEE and PEE(4).

000 x g for 30 minutes at the same temperature. Supernatant solutions were collected and kept at either 1°C, if the extract was to be used for immediate assay, or stored at –25°C. Storage at –25°C for several weeks had no effect on measured enzyme activity. *Esterase Assays.* Esterase activity was determined at 30°C in reaction solutions of ionic strength 0.6 using ATEE, TEE, and PEE as substrates(2,4,5). Hydrolysis of the non-acylated compounds was measured at pH 6.5; ATEE hydrolysis was measured at pH 8.0. Incubation of enzyme extracts with AIn preparation to inhibit Proteinase A or possible Proteinase A-type activity was executed under standardized conditions(2). Esterase activity was expressed as zero order reaction constant, k_0, defined as μmoles substrate hydrolyzed/minute/ml extract. *Proteinase assays.* Hydrolysis of alkali-denatured casein was determined after exposure of enzyme extracts to conditions which, in rat skin extracts, permit either activation or inactivation of Proteinase C(1-3). When *activation conditions* of assay were used, the extract was exposed to ionic strength 1.6 and 1°C before addition to substrate. After such treatment, Proteinase C will exhibit maximal activity. When *inactivation conditions* of assay were used, the enzyme solution was exposed to ionic strength 0.8 for approximately 6 hours at 1°C before assay. This type of enzyme pre-treatment permits association of Proteinase C with its inhibitor and is reflected by decrease in proteolytic activity. In both assay procedures, the enzyme-substrate reaction solution was at pH 7.5 and ionic strength 0.8. The temperature was 35°C. Activity was expressed as optical density change at 280 mμ/minute/ml of extract as determined under standard conditions(1,2). Protein was determined by the method of Lowry *et al.*(16), using commercial preparation‖ as the protein standard. All data obtained, except where indicated, represent mean value of 3 different assays with the attendant standard deviation, each assay being performed on the extract derived from the skin of a separate animal.

Results. The esterase and proteolytic activity of extracts from various animal skins are given in the Table. Activity towards ATEE was present in only rat, guinea pig, sheep, and monkey skin extracts (*cf.* Column I). Incubation of extracts possessing ATEEase activity with the AIn preparation produced inhibition of such activity in only rat skin extracts (compare Columns I and II). A slight potentiation of sheep ATEEase activity was noted by such treatment. Since the Proteinase A of rat skin extracts can be inhibited by AIn preparation, these results indicate that comparable activity is absent in skins of other animals tested. The AIn non-inhibitable ATEEase activity (*cf.* Column II) simulates activity of the A_1-esterase of rat skin extracts. The non-detection of a Pro-

‖ Protein Standard, Ampagent, Aloe Scientific.

teinase A-type enzyme in extracts other than those derived from the rat may be due either to its absence or to its existence in an inactive state. Evidence that Proteinase A exists partly in an inactive state in extracts of rat skin acetone powder(5) and in fresh rat skin extracts (unpublished results) has been obtained and such may be the case in other skin extracts.

Enzyme activity capable of TEE hydrolysis was somewhat more prevalent with only extracts of cow and chicken skin being devoid of activity toward this substrate (*cf.* Column III). However, hydrolysis of TEE by enzyme preparations apparently lacking an enzyme comparable to Proteinase A does not necessarily imply the existence of a rat skin-type A_2-esterase in such extracts. As mentioned above, the A_2-esterase hydrolyzes TEE and PEE at equal rates. Examination of the Table (Column IV) shows that the ratio of activity toward PEE and TEE is essentially unity only for the rat skin extract. In all other cases, TEE and PEE are hydrolyzed at different rates. In an attempt to determine if a single enzyme catalyzes hydrolysis of both TEE and PEE, as is true with the A_2-esterase in rat skin extracts, the enzyme preparations were stored at −25°C for 2½ months. PEEase to TEEase activity ratios remained constant. Likewise, heating for 10 minutes at 55°C, although producing a decrease in activity toward these 2 substrates, left the activity ratio unaltered. Although such evidence is equivocal, the data are not inconsistent with the viewpoint that a single enzyme in each extract catalyzes the hydrolysis of both TEE and PEE. The structural similarity of these 2 substrates strengthens this possibility. Therefore, despite the difference in TEE and PEE hydrolysis rates by extracts of skin other than the rat, the data suggest that an enzyme with a substrate specificity similar to the A_2-esterase is present in guinea pig, rabbit, hog, sheep, and monkey skin extracts. It should be mentioned that in 2 samples of human skin tested (data not given in the Table), one from abdominal area of a negro adult female and the other from the breast of a white adult female, only the extract from the former contained ATEEase activity and it was not inhibitable by the AIn preparation. Both samples were devoid of activity towards TEE.

Surprisingly, only extracts of rat, guinea pig, and monkey skin contained proteinase activity, and in all cases, exposure of enzyme preparations to *inactivation conditions* prior to assay led to decreased activity (compare Columns V and VI). Admittedly, the decrease in activity of guinea pig extract was slight but, as with rat and monkey skin extracts, it was completely reactivated upon exposure to *activation conditions*. Thus, extracts of monkey skin and, to a slight extent, of guinea pig skin, contain proteolytic activity capable of ionic strength-dependent reversible inactivation, *i.e.*, activity comparable to Proteinase C. The data for all monkey skin proteinase determinations have been presented separately due to variation of results obtained with each animal.

Summary. Fresh skin extracts of various animals have been surveyed for ability to hydrolyze N-acetyl-L-tyrosine ethyl ester, L-tyrosine ethyl ester, L-phenylalanine ethyl ester, and casein. The data obtained have been interpreted in terms of conformity to results previously obtained with extracts of rat skin acetone powder.

ADDENDUM. Since the preparation of this manuscript, a comprehensive review by Ungar, G., and Hayashi, H. (*Ann. Allergy,* 1958, v16, 542) has appeared. This review summarizes the experimental evidence for the role of enzymatic mechanisms in antigen-antibody reactions.

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Intraocular Grafts of Embryonic Sexual Ducts of the Rat.* (24554)

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The guiding hypothesis behind much of the investigation pertaining to differentiation of sexual ducts in vertebrates calls for a response of the ducts to secretions provided by the developing gonads. Accordingly, developing testes provide materials which selectively promote elaboration of wolffian ducts while suppressing the oviducts; developing ovaries do the converse. Generally speaking, this 'modus operandi' appears to prevail, yet some workers(1,2,3) incline to the view that in mammals only the testes are concerned in duct differentiation while another(4,5) feels development of the reproductive tract is not controlled by the gonads at all. With these alternatives in mind, it is believed that a report of some observations on behavior of gonads and sexual ducts of the embryo rat interplanted to the anterior chamber of the eye may be of interest. The observations derive from 230 grafts prepared for earlier studies on gonads and rete tubules(8,9). The general procedures in preparing this material have been described. It should be added, however, that not all two hundred odd grafts included the primordial sex ducts; thus, this report is based on a lesser group of 67 relevant cases selected from the total pool. Specific details as to ages of donor embryos, constitution and survival of grafts and other pertinent data will be given as these cases are described. A complete series of "normal stages" of rat embryos has been available as standard of reference. A necessary first step towards interpretation of the grafts is a brief review of the time-table of normal embryogeny of the parts concerned (6,7). The wolffian ducts split directly off the top of the nephrogenic ridge, beginning 10th day postcoitum, then progressively project themselves caudad by free terminal growth. They contact the wall of the cloaca during 11th day and 1 day later open thereto. By the 19th day, in females, wolffian ducts have disappeared. The first mesonephric tubules appear early on 11th day. A maximum number of 15 to 18 pairs of tubules is reached near the end of 12th day. Of these, all disappear by the end of 16th day except the 3 most anterior pairs which are retained as epigonadal elements. In contrast to the early appearing wolffian ducts, the oviducts do not arise until the latter half of 13th day and do not open to cloaca until 2 days later. They disappear in males by the end of 19th day. As for the gonads, those destined to be testes begin to assume their characteristic structure towards the close of 13th day; prospective ovaries do not express themselves until the 16th day. It follows from this listing that embryos of 14 days, plus or minus a few hours, present prospective ducts and epigonadal elements for both sexes and gonads of one sexual prospect or the other. Moreover, the presence of the opposite elements in grafts of a given sex residing in the eye chamber for periods which would make them equivalent to term or postpartum ages is indicative of departure from their normal fate. The grafts to be considered all derived from embryos ranging in age from 13½ to 14¼ days postcoitum and are of 2 types. Fig. 1 is intended to portray these. In one series, the urogenital

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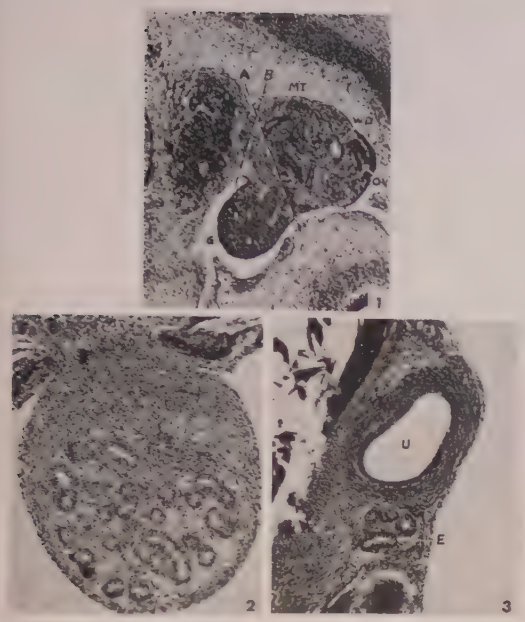

FIG. 1. Urogenital system of 14-day rat embryo. Line "A" shows plane of separation of mesonephric ridge; line "B" of urogenital ridge. MT, mesonephric tubules; WD, wolffian duct; OV, oviduct; G, gonad. ×35.

FIG. 2. Epididymis in graft of mesonephros. Theoretical postpartum age, 22 days. ×38.

FIG. 3. Graft of mesonephros at theoretical postpartum age of 10 days. E, epididymis; U, uterus. ×38.

area was split along the line "A" to provide a strip of tissue consisting solely of mesonephros and adjacent wolffian duct and oviduct. In the other series the entire urogenital primordium was detached along line "B". In other words, the 2 series provide tests of differentiating capacities of the oviduct and the wolffian duct with and without the gonads. The hosts were both male and female and ranged in age from 2 months to a year. Performance of the grafts was in no way correlated with age and sex of hosts, a point which will be considered elsewhere.

Results. Grafts of mesonephric ridge. Seventeen grafts comprise this series. Seven of them remained in the eye chamber until they reached an age equivalent to postpartum age of 3½ days. The remaining 10 were permitted to attain theoretical postpartum ages ranging from 10 to 22 days, averaging 12+ days. Of the 17 interplants, 12 (70%) survived to produce an assortment of structures; the remaining 5 regressed to a vascular stroma. In all 12 surviving grafts, vas deferens and vasa efferentia (epididymis) appeared to a variable degree (Fig. 2). The number of cases is too small to provide any basis for calculation of the genetic sex of the embryos which provided the grafts. It is conceivable that all 12 were prospective males and thus the presence of male ducts is only a manifestation of normal prospects. There are two related circumstances to be noted. Firstly, in 5 of these 12 cases the host animals were females. In fact, the particularly favorable case chosen for illustration (Fig. 2) is a graft which reached a theoretical postpartum age of 22 days in the eye chamber of a female host and in so doing produced a male duct system fully as elaborate as that of a normal male of this age. Secondly, in 3 grafts a well developed oviduct and uterus also appear (Fig. 3). Two of these developed in male hosts, the other in a female. The case illustrated involved a male host and has a theoretical postpartum age of 10½ days. The epididymis is less elaborate than in some other grafts, but still well formed; the uterus has a diameter approaching that in a normal female, but differs from the normal in being devoid of glands.

These results suggest that in the absence of associated gonads, both male and female ducts are capable of differentiation. Keeping in mind that the grafts acquire a blood supply from the irises of the hosts, it is obvious that the hormonal milieu provided by the host exercises no selective influence on the ducts. As to the lesser frequency of appearance of female ducts, these possible explanations have to be considered. The 3 instances of female ducts may indicate these were the only ones originating from genetically female embryos. If so, then one must account for simultaneous occurrence of male elements. Conversely, as already suggested, all grafts may have been male in origin which calls for an accounting for the presence of any female parts at all. It is even conceivable that all grafts may have been genetic females or distributed between male and female in some other proportion. Since the number of cases is too small for any

analysis of probabilities here, one can only point to the fact that the intraocular environment appears to favor differentiation towards maleness(9). But further information is provided by the second series of grafts.

Grafts of the urogenital ridge include fifty grafts. Of these, 6 were resorbed and 7 were lost through infection or death of host, leaving 37 for analysis. Theoretical postpartum ages ranged from 6 to 59 days with an average of 20+ days. The bulk lay in 12 to 22 day range, with fewer at extremes. An analysis of these grafts (in conjunction with others of a different type) with respect to performance of gonads has been reported(9). It need only be recorded here that of the 37 grafts, 4 consisted solely of vascular stroma and 5 presented stroma and various non-gonadal tissues only. The remaining 28 showed gonadal differentiation as follows: 26 testes, 1 ovary and 1 ovotestis. The 26 cases of testes in turn fall into 2 groups: 18 conventional testes and 8 possessing structures which have been termed "converted medullary tubules." Evidence for the conclusion that the latter should be interpreted as having been zygotic female in origin has been given(9). If one assumes that the 9 cases wherein only stroma and/or non-gonadal tissues appeared were female, by no means a necessary assumption, and adds these plus the 2 cases of ovary and ovotestis to the 8 cases of converted medullary tubules, there results a total of 19 judged zygotic female alongside 18 judged male.

This census of the status of the gonads in the grafts has been recorded to provide a reference base for interpretation of such sex ducts as also appeared. As in the previous series, both male ducts (vas deferens and vasa efferentia) and female ducts (oviduct and uterus) appeared. The following summarizes their occurrence with reference to status of the gonad.

Male ducts + testis	4
Female ducts + testis	4
Male ducts + ovary or ovotestis	2
Female ducts + ovary or ovotestis	0
Male ducts + converted medullary tubules	4
Female ducts + converted medullary tubules	0
Male and female ducts + converted medullary tubules	1
Male ducts without gonads	1
Female ducts without gonads	1
Testis without ducts	10
Converted medullary tubules without ducts	3
Total	30

The above summary accounts for 30 of the 37 recovered grafts. It has already been noted that 4 consisted of stroma alone; the remaining 3 presented no reproductive structures of any kind, but did contain adrenal cortex and cartilage. Of 30 cases, the obvious point to be noted is that sex ducts of one kind or another appear in only 17 of them. This is in striking contrast to their more frequent appearance, percentagewise, in grafts of the mesonephric ridge alone. One might expect them to survive and differentiate at least as well in association with gonads, yet that they do not, can only be a matter for speculation. It is possible, of course, that presence of gonads is actually deleterious to their development, and the 2 cases of duct development in the absence of the gonads would bear this out. Yet where the ducts do occur in the company of gonads, their development is just as complete as in the absence of gonads; in normal ontogeny, ducts and gonads accompany each other.

A second general point is that there is no correlation between presence or absence or type of duct and sex of host. Of the 4 instances of male ducts + testis, 3 were in male hosts, the other in a female; of 4 female ducts + testis, all 4 were in male hosts; of 4 male ducts + converted tubules, 2 were in males and 2 in females; the ovary + male ducts was in a male, the ovotestis + male ducts in a female; and in the case of male and female ducts together + converted tubules, the host was male.

The very randomness of association between duct types and gonad types has its positive and negative aspects. On the negative side, there is no consistency or pattern of performance exhibited by the ducts. Female ducts appear in conjunction with testes as often as male ducts. While it is true, with the exception of the case of simultaneous appearance of both ducts, that only male ducts ap-

pear in conjunction with converted tubules, which would suggest an influence towards maleness emanating from this type of testis, it also happens that only male ducts appear on the rare occasions of the differntiation of ovarian tissues, the reverse of what one would expect if the gonads are really exercising any influence. There is, of course, no evidence as to whether the gonads in these grafts produce endocrine substances at all. If they do, there is no consistency of endocrine effect upon the ducts; if they do not, the independence of the ducts is self-evident. On the positive side, this randomness of differentiation of the ducts coupled with the fact that good gonad differentiation as often as not fails to elicit or support any duct differentiation whatsoever suggests the essential independence of the ducts.

Conclusion. Reviews(11,12) of the vast literature on gonadal control of embryonic differentiation of sexual ducts in mammals reveal 3 general viewpoints: (a) that the developing testes and ovaries produce sex hormones and that each has 2 opposing actions, stimulation of ducts of corresponding sex and inhibition of ducts of opposite six; (b) that the testes produce hormone which stimulates male parts while suppressing the female, but the ovaries do not influence sexual differentiation; (c) that sexual differentiation is not governed by gonad hormones. The results here submitted, namely, absence of correlation between duct development and hormonal environment provided by host and the similar lack of correlation between ducts and accompanying gonads, support the third view. This conclusion conforms in general to that reached by Bronski(10).

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Release of Norepinephrine from the Isolated Heart.*† (24555)

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Hoffmann *et al.*(1) reported the presence of an epinephrine-like substance in the perfusate of isolated mammalian heart treated with acetylcholine or nicotine. Similar observations were made by McDowell(2). More recently Paasonen and Krayer noted a marked decrease of norepinephrine content of mammalian heart treated with reserpine(3). These observations were based on bioassay technics. The present report concerns chemical identification and assay of biologically active agents liberated from the isolated heart during the interval of stimulation following administration of acetylcholine.

Methods and materials. Isolated rabbit hearts were perfused by means of a modified Langendorff apparatus with oxygenated

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Ringer-Locke solution containing 1 mg atropine/liter of perfusion fluid. Test doses of acetylcholine (25 to 200 μg) were introduced into the perfusion fluid at intervals. Perfusates from the heart were collected before administration of acetylcholine and during interval of stimulation which followed. They were analyzed fluorimetrically for their catechol amine content(4). Also, fluorescence emission spectra were recorded by means of Aminco-Bowman spectrophotofluorometer. Biologic activity of the perfusate was assessed by means of its effect on isolated segments of rabbit intestine. More decisive indications of biologic potency were obtained by injections of appropriately treated perfusates into a dog prepared for recording blood pressure and heart contractile force(5).

Results. Large doses of acetylcholine (25 to 200 μg) increased the amplitude of contraction of the isolated perfused heart and correspondingly produced substantial increments in concentration of norepinephrine in the perfusion fluid. In general, increasingly larger doses were required to elicit subsequent comparable degrees of stimulation. Positive inotropic effects were consistently obtained in 18 hearts in which 83 administrations of acetylcholine were made. In 6 of these preparations chemical analysis was done on 14 separate samples taken during stimulation. As contrasted with control determinations there were constant and substantial increases in the norepinephrine fraction with essentially no change in the epinephrine fraction. Mean levels of maximal norepinephrine values were about 10 times control figures (Table I). As recorded by Aminco-Bowman spectrophotofluorometer, the fluorescence emission spectrum of condensation products of rabbit heart perfusate with ethylenediamine was identical with emission spectrum of an authentic mixture of norepinephrine and epinephrine containing the same calculated amounts of catechol amines found in the perfusate. Biologic activity of perfusates collected during acetylcholine stimulation was clearly manifested on the small intestine and on the cardiovascular system. When added to the bath of isolated intestinal segments, marked inhibition of motor function was observed (Fig. 1); when injected intravenously into a dog prepared for recording blood pressure and heart contractile force, substantial increments were noted in these 2 parameters (Fig. 1). In the latter instance, pooled perfusates collected during successive periods of stimulation were adsorbed on a column of aluminum oxide and subsequently eluted with weak acetic acid. This eluate was then injected into the test animal. Increases in cardiac contractility and in blood pressure were similar in character and in the same range as those seen after intravenous administration of epinephrine 0.4 μg/kg.

TABLE I. Catechol Amine Content of Perfusion Fluid from 6 Isolated Rabbit Hearts.

Control		After acetylcholine max values	
Norepinephrine	Epinephrine	Norepinephrine	Epinephrine
μg/l			
1.6	6.2	12.7	4.9
4.0	4.5	31.4	1.7
2.6	1.5	36.2	4.2
8.3	1.4	12.8	2.1
.3	1.0	6.7	1.9
.8	.3	32.4	2.0
Mean 2.9	2.5	22.0	2.8

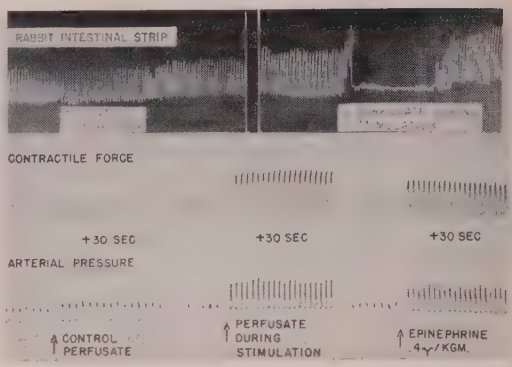

FIG. 1. Upper tracing: contractions of isolated rabbit intestinal segment suspended in Ringer solution. Perfusates collected from rabbit heart before (control) and during acetylcholine stimulation. Middle and lower tracings: effects of extracts of rabbit heart perfusates on heart contractile force and arterial blood pressure of a test animal (open-chest dog preparation).

Discussion. Increasing emphasis is being accorded the autonomic nervous system in controlling heart function. This control may

be modified by release of sympathomimetic substances from specialized tissue within the heart. The present report suggests the presence of ganglionic structures in the heart since the bulk of the sympathomimetic agent in the perfusate was norepinephrine. Schmitthenner *et al.*(6) concluded that increases in coronary blood flow, cardiac oxygen consumption, and left ventricular work elicited by nicotine infusions were due predominantly to stimulation of sympathetic ganglia of the heart with liberation of norepinephrine and epinephrine locally. West *et al.*(7) demonstrated that intracoronary arterial injections of nicotine produced increases in myocardial contractility and coronary blood flow qualitatively similar to effects produced by intracoronary injections of norepinephrine and epinephrine. Recently, Burn and Rand(8) demonstrated that isolated atria from rabbits pretreated with reserpine no longer exhibited positive inotropic and chronotropic effects when tested with nicotine. Recognizing the ability of reserpine to deplete norepinephrine stores of cardiac tissue, these authors concluded the stimulant action of nicotine was due to release of epinephrine and norepinephrine from the heart. The possibility of the existence of chromaffin tissue in the heart should not be excluded, however, since it would probably be stimulated by acetylcholine in the same manner as the adrenal medulla. If such were the case, however, it would be necessary to postulate a difference in composition of the chromaffin tissue at the 2 sites since the hormone of the adrenal medulla is predominantly epinephrine and the hormone liberated from the heart as demonstrated here is predominantly norepinephrine.

Activity of cardiac drugs may be dependent to some extent upon release of adrenergic amines within the heart. These amines may, likewise, play an important role in coronary artery disease. The significance of epinephrine and related compounds in heart muscle has been reviewed by Raab(9). Recent work in our laboratory has revealed elevated plasma levels of catechol amines in patients with angina pectoris after exercise and in myocardial infarction. Under such circumstances the damaged heart might conceivably contribute to these increments in circulating pressor amines.

Summary. Acetylcholine produces a prompt positive inotropic effect on the isolated atropinized heart. Norepinephrine is the agent largely responsible for this action as judged by biological and chemical assay of perfusates collected during the period of stimulation.

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Inhibition of Aminonucleoside Nephrosis by Adenine.* (24556)

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Repeated injection of 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl) purine, (hereinafter referred to as aminonucleoside)†, a derivative of the antibiotic, Puromycin, produces a disorder in rats characterized by hyperlipemia, azotemia, hypoproteinemia and proteinuria(1-5). This disorder resembles the nephrotic disease of children and the experimental disease produced in rats by injection of a rabbit anti-rat kidney serum(6-9). Several metabolic manifestations of the heteronephrotoxic type of nephritis have been reported. These include: (a) impaired phosphorylation during anaerobic glycolysis using kidney slices(10), (b) grossly normal carbohydrate metabolism(11), (c) primary protein loss followed by an increased protein turnover, increased utilization of carbohydrate and accelerated amino acid metabolism (12), (d) mobilization of body fat following the primary protein loss(13). Since nephrosis is induced by minute amounts of aminonucleoside, and as it is related structurally to adenine and adenosine, it seemed likely that this compound could be acting as a metabolic antagonist in some phase of purine metabolism including reactions involving ATP. The aminonucleoside has an anti-trypanosomal effect in mice infected with *Trypanosoma equiperdium*(14). This effect is partially reversed by a number of synthetic purine derivatives and by adenine but not by adenosine. Additionally, the aminonucleoside is effective against other organisms, and various purines serve to reverse its growth inhibiting activity(15). As the compound appears to be a metabolic antagonist whose activity is reversed by purines in lower forms, it seemed desirable to determine whether it acted similarly in mammalian species. The experiments to be described were designed to determine the effect of adenine and adenosine on the production of the aminonucleoside-induced nephrotic syndrome in the rat, with the hope that the information gained would be of value in elucidation of the basic etiology of heteronephrotoxic kidney disease and lipoid nephrosis of children.

Materials and methods. 58 female Sherman strain rats ranging 70 to 123 g were divided into 5 groups. Each rat received the following compounds by subcutaneous injection in amounts indicated/g of body weight/day. Those in Group 1 received 0.015 mg of aminonucleoside. Those in Group 2 received 0.015 mg of aminonucleoside plus 0.056 mg of adenine suspension. Those in Group 3 received 0.015 mg of aminonucleoside plus 0.8 mg of adenosine suspension. Those in Group 4 received 0.056 mg of adenine suspension and those in Group 5 received 0.8 mg of adenosine suspension. The animals in Groups 2 and 3 received adenine or adenosine just prior to the aminonucleoside at a separate subcutaneous site. The animals were held for 14 or 15 days during which time they were given 10 injections, the last on day of sacrifice and the day preceding sacrifice. On final day of experiment urines were collected, tested for presence of protein by heat and acetic acid test and the results gauged on a 0 to 4+ scale. The animals were anesthetized and a midline laparotomy incision was made. When present, the amount of ascites was measured by withdrawing as much of fluid as possible into a small graduate. The animals were killed by exsanguination and total serum lipids determined by the turbidometric method of de la Huerga *et al.*(16). Kidney samples were taken for sectioning and staining with H&E and PAS. The sections to

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TABLE I. Comparison of Mean Values of Ascites, Proteinuria and Total Serum Lipids in Rats Which Received Aminonucleoside with and without Addition of a Purine Derivative.

Group	Compound(s) inj.	No. of rats	ml of ascites	% with 3+ or 4+ proteinuria	mg % total serum lipid
1	Aminonucleoside	11	13	91	1005
2	" + adenine	12	0	25	592
3	" + adenosine	12	7	92	750
4	Adenine	11	0	0	300
5	Adenosine	12	0	17	286
	None	12			338

be stained with H&E were cut at 7 μ and those to be stained with PAS were cut at 4-5 μ. Comparative statistical studies were made between appropriate groups. The *t* test was used to compare data for lipid levels and amounts of ascitic fluid. A value was considered to be statistically significant when its P value was .05 or less. The chi square test was used to evaluate the significance of proteinuria data taking into account the 5 values ranging from 0 to 4+.

Results. Comparisons of data obtained from each group of rats and from 12 normal animals of similar weight are shown in Tables I and II. When compared to results obtained with rats which received aminonucleoside alone, concomitant administration of adenine with the aminonucleoside 1) prevented accumulation of ascitic fluid, 2) resulted in a highly significant decrease in number of animals with 3+ or 4+ proteinuria and 3) led to markedly lower serum lipid levels. Concomitant administration of larger amounts of the more soluble adenosine (over 8 times as much adenosine as adenine on a molar basis) with the aminonucleoside, was not followed by similar manifestations of reversal of toxicity of the aminonucleoside. At the level of adenine administered (molar ratio of adenine to aminonucleoside 8:1) reversal of toxicity was not complete. However, it did not seem feasible to increase the molar ratio of adenine to aminonucleoside, since in preliminary experiments adimnistration of larger amounts of adenine alone led to toxic manifestations. Neither adenosine nor adenine were totally innocuous compounds at the levels used, inasmuch as administration of either compound was followed by a significant change in degree of proteinuria. Administration of adenine alone did not cause 3+ or 4+ proteinuria but was followed by more than the expected incidence of 1+ and 2+ reactions.

TABLE II. Statistical Comparison of Data.

Group	Groups compared	Total serum lipids Diff. ± S.E.	Total serum lipids P value	Proteinuria P value
1	Aminonucleoside			
2	Aminonucleoside + adenine	423 ± 123	<.01	<.01
2	Aminonucleoside + adenine			
4	Adenine	289 ± 71	<.01	>.05
1	Aminonucleoside			
3	Aminonucleoside + adenosine	255 ± 129	>.05	>.05
3	Aminonucleoside + adenosine			
5	Adenosine	470 ± 82	<.01	<.01

Serum lipid levels of animals which received aminonucleoside plus adenine were significantly lower than those of animals which received only aminonucleoside. Levels in animals which received both compounds however, were significantly higher than those in animals which received adenine alone. Thus the aminonucleoside effect was not totally blocked. Lipid levels in serum of animals treated with adenine alone, were not significantly different from those of normal animals.

Administration of adenosine with aminonucleoside did not result in significant differences in lipid levels from those of animals which received aminonucleoside alone. It is interesting that injection of adenosine alone resulted in significantly lower lipid values than those observed in normal animals.

Administration of aminonucleoside produced an injury of the basement membrane,

manifested by a deposit of PAS positive material. Either adenine or adenosine also caused early changes in basement membrane characterized by splitting and fraying. The appearance of basement membrane was not altered by addition of either adenine or adenosine to aminonucleoside. The PAS positive material deposited in the glomerulus did not appear as dense as that seen in the nephrotoxic nephritis, however, the tubular lumina contained prominent PAS positive casts.

Discussion. The data herein presented provide evidence that the mode of action of aminonucleoside in production of the nephrotic syndrome in the rat is that of an antimetabolite. Reversal of toxicity of the compound by adenine would implicate a block in some phase of nucleotide or nucleic acid metabolism. The question is thus raised as to whether other types of nephrotoxic disease may have a similar etiology, *i.e.,* deranged nucleic acid or nucleotide metabolism. In a yeast system it has been observed(17) that aminonucleoside inhibited ATP formation. The negative results obtained with adenosine do not necessarily exclude the possibility of a defect in the phosphorylation mechanism. Adenosine deaminase has been found to be very active in mammalian tissues(18) and it is possible that rapid conversion of adenosine to inosine might account for its lack of effect in the present study. Brown *et al.*(19) noted greater incorporation of exogenous adenine than of adenosine into the nucleic acids of the rat and proposed that the route for incorporation of the free base is different from that of nucleotides synthesized *de novo.* Buchanan (20) has related that aminonucleoside does not inhibit any of the enzyme systems involved in the *de novo* synthesis of purine derivatives.

The intraglomerular deposits of PAS positive material seen here corroborate the findings of Frenk *et al.*(1) but are at variance with the report of Fisher *et al.*(4). In the experiments of the latter workers injections of the aminonucleoside were stopped on day 9-11 and the animals were held until day 14. The possibility exists that in the experiments of Fisher the PAS positive material had been present at the end of injection period and had decreased in amount in the 3-5 day period immediately prior to sacrifice. This would be in accord with the observations of Frenk *et al.* (1) that structural alterations are reversible in animals receiving 5-20 consecutive daily injections of the aminonucleoside. It should also be noted in considering the results of these two authors and those presented here, that 3 different strains of rats were used. Thus genetic differences in susceptibility of the mucopolysaccharides of the basement membrane to injury could have been responsible for the discrepancy in results. The fact that morphological alleviation of the disease lagged behind the functional improvement may have been due to the masking effect of the adenine which itself gave rise to early basement membrane changes.

Summary. Studies were made of the effects of administration of adenine and adenosine on nephrosis of rats induced by injection of aminonucleoside obtained from Puromycin. Adenine partially reversed nephrotoxic activity of the aminonucleoside, while adenosine did not. These findings have been interpreted as evidence that aminonucleoside acts as an antimetabolic agent in some phase of nucleotide or nucleic acid metabolism in production of the nephrotic state in the rat.

The technical assistance of Antoinette C. Bonfilio is gratefully acknowledged.

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Inhibition of Experimental Tumors by Orthophenylenediamine (OPDA). (24557)

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The presence in animals of certain tumors has been observed to provide either biochemical protection or liability in terms of survival time for the host when challenged with a lethal dose of specific chemical agents such as the phenylenediamines(1,2). The Cloudman S91 mouse melanoma protects its host against the acute toxicity of paraphenylenediamine (PPDA) whereas the same tumor is a disadvantage to the host when the challenging compound is orthophenylenediamine (OPDA). With the above experimental design this phenomenon extended to other combinations of tumor and compound variously to the advantage, disadvantage, or neither for the host. In possible correlation, it was also observed that PPDA combines with certain pigmented tumor constitutents *in vitro* as determined by oxygen-consuming reactions in the Warburg apparatus when added to melanoma tissue, urine, blood plasma, or pure dihydroxyphenylalanine (DOPA)(3,4,5). Further pursuit of these phenomena led to chemotherapeutic testing of phenylenediamines against melanoma and other tumors. The results with the ortho isomer, which is the least toxic and most effective, are reported here with a variety of transplanted mouse and hamster experimental tumors.

Materials and methods. Several inbred and hybrid strains of mice were employed for carrying the tumor types studied. Most melanoma experiments utilized the Cloudman S91 (6) although the Harding-Passey(7), the Fortner hamster melanoma(8), and several non-melanoma tumors were used for comparative purposes. The transplanted hamster melanoma is designated as Melanotic Melanoma No. 2 (H.M.M. #2) and originated "spontaneously" in a Syrian golden hamster. The histological and biological characteristics have been described(8,9). The Cloudman S91 mouse melanoma was carried in both male and female DBA/2 hybrid mice. The Harding-Passey melanoma and the Ehrlich car-

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cinoma(10) were grown in male Hauschka ICR Swiss albino mice. The Fortner hamster melanoma was carried in Syrian golden hamsters obtained from commercial stock. Tumors were transplanted by subcutaneous inoculation of 0.1 ml of 10 or 20% tissue suspension into the right hip. All measurements of tumors were with calipers in 3 dimensions. Volumes were determined by multiplying length by width times thickness, which gave acceptable correlation with tumor weights. Weights of individual animals were obtained with a Mettler single pan balance with precision of $\pm$ 0.03 g and are expressed in the Figures as calculated group averages. Orthophenylenediamine (OPDA) was used as the free base. Most commercial preparations originally obtained were impure or contained oxidized products and, although they possessed antitumor activity, efforts were made to further purify such products to reduce nonspecific toxicity. A satisfactory commercial preparation of OPDA (free base) is now available.† Solutions for animal injections were prepared fresh daily in distilled water or 0.5% CMC (sodium carboxymethylcellulose) solution. Unless otherwise indicated, all animals were injected intraperitoneally once a day, 5 days/week.

Results. Fig. 1 illustrates the effect of OPDA on tumor growth and total mouse weight when administered to animals with well-established transplanted melanomas. The tumor in this experiment was the Cloudman S91 pigmented melanoma which had been implanted 17 days prior to start of treatment. The initial intraperitoneal dose was 100 mg/kg/day for 5 successive days with an increase the second week to 150 mg/kg and finally to 200 mg/kg maintained for over 70 days. The injection schedule is shown in Fig. 1. It may be seen that treated tumors continued to grow slowly under this dose schedule for approximately 30 days with a drug-induced inhibition of about 75%. At this point when untreated control animals were dying from their extensive tumors a significant decrease in size of treated tumors began to occur. This slow growth and regression of the tumors is also reflected in total weights of animals. Each point on the graph is the average of the surviving animals with tumors, in the case of tumor volume curves and of total number of animals in each group in the weight curves. There were 10 mice in each group at beginning of experiment with a gradual decrease to zero survivals at 30 days in saline-injected controls and to 2 in the treated group at 70 days.

The effect of OPDA on growth of the Harding-Passey melanoma, when administered at constant dose level of 200 mg/kg/day 5 times a week, is illustrated in Fig. 2. In this experiment the compound was administered subcutaneously rather than intraperitoneally, which seemed to inhibit as effectively or slightly better than i.p. injections at this dose level with the Harding-Passey melanoma. Slightly more than 80% tumor growth inhibition was obtained after 40 days treatment. As also illustrated in Fig. 2, there was a steady weight gain in the treated animals, which suggested that this dose level was not the maximum that could be tolerated.

Fig. 3 summarizes the results of OPDA treatment of a rapidly growing, metastasizing hamster melanoma. Tumor growth inhibition was approximately 85% after 40 days of treatment with 14 doses of 200 mg/kg and 15 doses of 250 mg/kg. It may also be seen that there was a steady weight gain in treated animals during this dosage regime. The added increase in weight of the untreated controls was due largely to tumor growth which eventually affected the animals' weight adversely as the tumors increased in size and metastatic development took place.

Fig. 4 illustrates the results of OPDA treatment at higher dose levels on the Ehrlich solid carcinoma. Treatment was started on one-day-old tumor transplants at a dose of 100 mg/kg. The second day it was increased to 150 mg/kg and, following a 2-day intermission, treatment was continued for 5 consecutive doses at 200 mg/kg followed again by a 2-day recess. On the fourteenth day the dose was raised to 300 mg/kg/day. This dose was then maintained 5 days a week until the thirty-fourth day at which time treatment was

† Abco Chem. Co., 68 Fleet St., Jersey City.

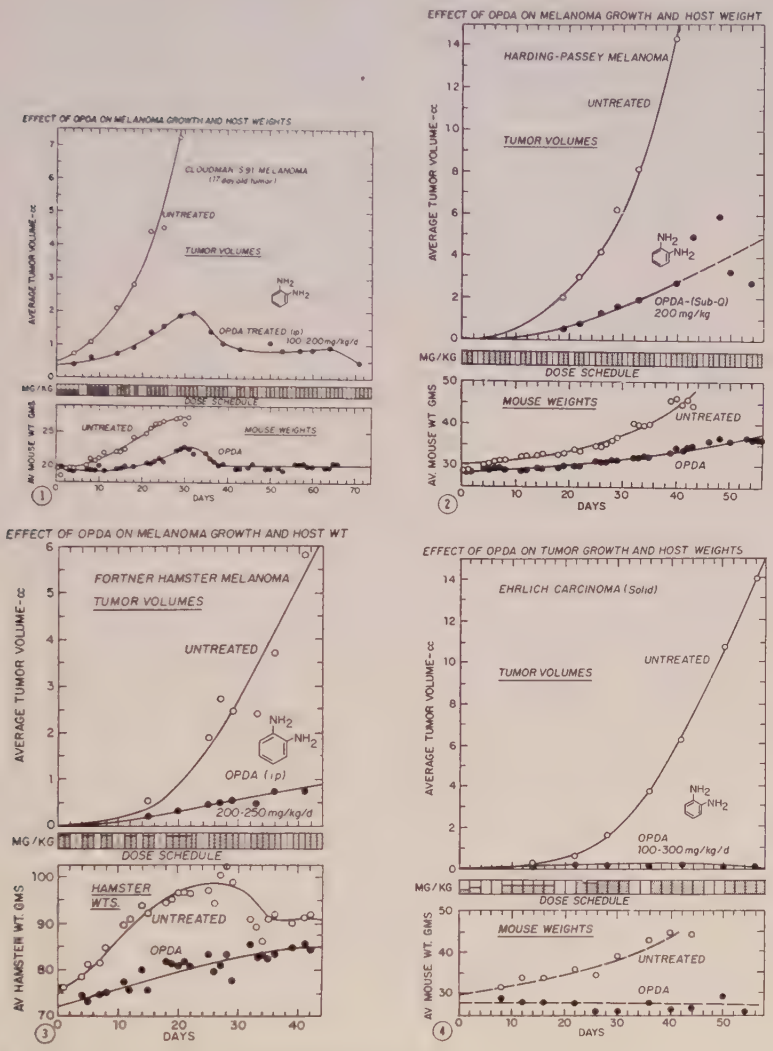

FIG. 1. Effect of OPDA (orthophenylenediamine) on melanoma growth and host wt. (Tumor: Cloudman S91 mouse melanoma, 17 days post implant in DBA/2 inbred mice. OPDA aqueous solution inj. i.p. at 100-200 mg/kg/day, controls inj. with physiological saline.)

FIG. 2. Effect of OPDA on Harding-Passey melanoma and host wt at constant dose. (Tumor 1 day old at start of treatment. OPDA-water solution, subcut. injections of 200 mg/kg/day.)

FIG. 3. Effect of OPDA on Fortner hamster melanoma and host wt. (Tumor: HMM-2, 1 day old at start of treatment. OPDA-water solution, 200-250 mg/kg/day, intraper. inj.)

FIG. 4. Effect of OPDA on the solid Ehrlich carcinoma and host wt. (Tumor 1 day old at start of treatment in Swiss ICR male mice. OPDA-CMC solution, 100-300 mg/kg/day, intraper. inj.)

discontinued. As illustrated by the plot of treated tumor volumes, over 95% tumor growth inhibition was obtained. The points plotted represent average tumor volume of tumor-bearing animals and do not include mice in the treated group in which the tumor failed to reach a measurable size. About half of the treated animals failed to develop tumors to the point where they could be measured. By the thirty-third day the tumors under treatment with OPDA (in CMC) had regressed to a point where they could no longer be measured. Animals in similar experiments have remained free of tumors for several

months and when rechallenged with a similar tumor tissue suspension on the opposite hip were resistant to the second implant.

OPDA has been submitted to the SKI tumor spectrum(11) where it was tested against a variety of 24 mouse, rat, and hamster tumors. A number of tumors showed inhibition within this test system and the results will be detailed elsewhere.

Discussion. The phenylenediamines are physiologically active compounds(12,13,14), and it is therefore a complex experimental problem to establish the mechanism of action of their antitumor properties. Their ability to combine in oxygen consuming reactions with certain catechol amines such as dopa and related products present in some pigmented tumors, is provocative but inconclusive evidence of their mode of action. This class of compounds has also been reported to have inhibitory effects against DPN-dependent enzyme systems(14). In testing the effect of ortho-, meta-, and paraphenylenediamine on respiration and glycolysis of Ehrlich ascites cells in manometric experiments, inhibition by all 3 isomers was observed in respiration but not in glycolysis under conditions of testing (unpublished). This suggests that DPN enzymes are not critically involved in the inhibition of Ehrlich carcinoma cells. It also indicates that aerobic respiration of the cell is more sensitive to these compounds than the anaerobic pathways. From the chemotherapeutic standpoint the structural simplicity of OPDA is of special interest and lends itself to simple chemical substitution and radioactive labeling for a systematic study of its action.

Summary. The antitumor effects of OPDA (orthophenylenediamine) on mouse and hamster melanomas and the Ehrlich solid carcinoma are reported. Complete inhibition of growth of the Ehrlich solid carcinoma was obtained as well as complete regression of some established and measurable tumors.

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Effect of Growth Hormone and Oxytocin Upon Milk Yield in the Lactating Rat.* (24558)

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It has been demonstrated on basis of milk yields that growth hormone (GH) causes an increase in milk secretion in cows(1,2), sheep, (3), and goats(4); while litter growth data suggested that it may be ineffective in rats (5). It is well known, however, that amount of milk removed during nursing or milking is related to quantity of oxytocin discharged from the neurohypophysis(6,7). It seemed of interest, therefore, to reinvestigate the role of

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TABLE I. Effect of GH and Oxytocin upon Milk Removal by Litters of 6 Young on Day 14 Postpartum.

Treatment	No. of lactating rats	Milk (g)	Litter (g)	Milk Litter (%)	Avg wt of– Mother (g)	Pituitary (mg/100 g)	Adrenal (mg/100 g)	Thyroid (mg/100 g)
Control	30	6.20	163.6	3.8 ± .29[1]	303.5	3.39	23.41	4.06
Control: 3-6 USP units /kg oxytocin IV on day 14	30	9.20	164.2	5.6 ± .14	308.9	3.38	23.57	4.04
Control: .1 USP unit/ kg oxytocin IV on day 14	25	9.81	172.6	5.7 ± .22[2]	299.1	3.42	22.96	3.74
GH: 1 mg/day from days 7-14	28	8.80	163.3	5.4 ± .32[3]	300.7	3.44	23.48	4.08
Idem + .1 USP unit/kg oxytocin IV on day 14	25	7.90	171.7	4.6 ± .10[4]	312.5	3.33	24.16	3.99
Idem, but .3 unit	25	10.80	176.9	6.1 ± .12[5]	316.2	3.29	22.18	4.11

Student's "t"	Probability
1–2,3,5	.005
1–4	.02
2–4	.005
2–5	.3
3–4	.01
3–5	.1
4–5	.005

GH in lactation in rats in terms of milk yields obtained during nursing with and without aid of oxytocin.

Materials and methods. Method of obtaining milk: Mature primiparous lactating rats of the Sprague-Dawley-Rolfsmeyer strain which had reached growth stasis were used. They were housed in individual cages under conditions of uniform temperature (78-80°F) in a room artificially illuminated during normal daylight hours and fed Purina Lab Chow and water *ad libitum*. Shortly after birth each litter was adjusted to 6 young and, whenever possible, were equally distributed as to sex. When 14 days of age, each litter was isolated from their mother for 10 hours. During this interval any milk present in stomachs of young is digested(8) and the mammary glands of mothers become turgid with milk. At the end of isolation, mother and young were reunited and young permitted to nurse 30 minutes. Seventy-eight lactating rats were injected subc. with growth hormone (GH)‡ at a dose of 1 mg/rat/day from days 7-13 of lactation. Twenty-eight GH-treated and 30 control lactating rats, half of which were similarly injected with saline, were assayed for milk yield in this manner. Fifty-five control and 50 GH-treated lactating rats were anaesthetized with Nembutal (45 mg/kg i.p.) 10-20 minutes prior to end of isolation. When completely anaesthetized, each mother was laid on her side and her litter replaced. Oxytocin‡ was then injected rapidly in a volume not exceeding .06 ml into the superior epigastric vein a few minutes after the young had been replaced and while they were actively sucking. After each litter had nursed for 30 minutes, they were removed, weighed, killed by decapitation and stomach contents removed and weighed to nearest 0.1 g. Weight of milk obtained is then expressed as percent litter body weight. Each mother also was killed shortly after nursing and her thyroid, adrenals, and pituitary glands rapidly removed, blotted on filter paper to remove surface moisture and weighed to nearest 0.1 mg. Daily body weights of each mother and her litter were recorded.

Results. Weight of milk, expressed as per cent litter body weight, obtained by litters of 30 untreated rats averaged 3.8% (Table I) with normal distribution. A significantly greater yield (5.7%), which represents an in-

‡ Kindly supplied by Dr. Irby Bunding, Armour Labs., Chicago.

TABLE II. Effect of GH upon Body Weight Gain of Lactating Rats and Their Litters from 6th-14th Days Postpartum. Litter weights at day 14 corrected for weight of milk obtained during 30 min. nursing.

Treatment	No. of rats	Avg wt (g) of Litter Day 6	Litter Day 14	Litter Gain	Litter % gain	Mother Day 6	Mother Day 14	Mother Gain	Mother % gain
Control	45	78.7	166.5	87.8	111.6 ± 2.5[1]	297.0	300.3	3.3	1.1 ± .36[3]
GH, subcut.	63	74.5	159.3	84.8	113.8 ± 2.9[2]	294.4	316.4	22.0	7.5 ± .12[4]

	Student's "t"	Probability
	1–2	>.2
	3–4	.001

crease of 50%, was obtained when .1 USP unit/kg oxytocin was injected i.v. into Nembutal anaesthetized mothers while the young were nursing. Variability in milk yield was reduced. No further increase in milk yield resulted when 3-6 USP units/kg oxytocin were administered (Table I). GH caused a significant increase in average yield of 42.1% but individual values showed more variability than controls. Milk yield obtained in GH-treated rats with aid of .1 USP unit/kg oxytocin was significantly less than obtained in control rats with the same dose of oxytocin. An increase to .3 USP unit/kg was necessary to elicit near maximum withdrawal of milk (6.1%) which represents a 60.5% increase over untreated controls and a 7% increase over oxytocin-injected controls. This was accompanied by further reduction in variability in milk yields. Percent weight gain of litters of GH-treated lactating rats from days 6-14 postpartum was insignificantly greater than for control litters (Table II). GH-treated mothers gained an average of 2.7 g/day during period of treatment which represents a significant percentage weight increase over .5 g/day gain for controls. Correlation of litter weight with milk yield of controls on day 14 indicated litter weight increased with increasing milk yield to where 7-8 g milk was obtained during 30 minutes nursing (Fig. 1). Greater yields were not reflected in a further increase in litter weight. GH did not cause a significant increase in the wet weight/100 g body weight of maternal pituitary, thyroid, or adrenal glands (Table I).

Discussion. A serious difficulty in lactational studies on rats has been the lack of satisfactory means of milking them and thereby evaluating milk secretion or removal. Lactational performance in rats generally has been inferred by litter growth and survival data whereby an alteration in growth is thought to mean that an alteration in milk secretion has occurred(9-13). This method has considerable merit in evaluating milk secretion in nutritionally or hormonally deficient animals. However, in a normal intact lactating animal, the young have a genetically inherent capacity for growth which cannot be exceeded. We question whether more intense milk secretion can produce litter weight gain in excess of this capacity since, in the present investigation milk yield and litter were correlated only to a point beyond which, though milk yield was greater, no parallel increase in litter weight was observed. Thus, in healthy animals, an increase in amount of milk obtained under standardized conditions, is suggested to mean an increase in milk secretion has occurred. On this basis, in the present study, GH evoked an increase in milk secretion of a similar magnitude as reported for other species(1-4) which, however, was not accompanied by a significant increase in litter weight. We had found previously that amount of oxytocin released due to 30 minute nursing stimuli on day 14 varied from less than .02 to .1 USP unit/kg (7). In the present study administration of .1 USP unit/kg oxytocin resulted in 50% higher yields with greater uniformity of individual values which suggests milk removal and, indirectly, milk secretion, of lactating rats is limited by amount of oxytocin discharged due to the stimulus of nursing. This level may be considered optimal since larger doses of oxytocin failed to evoke greater milk withdrawal. It is suggested GH at level em-

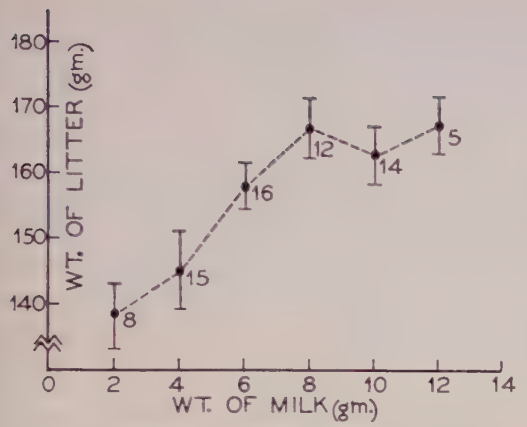

FIG. 1. Correlation of amount of milk obtained by litters of 6 young during 30 min. nursing following isolation of mother and young for 10 hr with weights of litters after nursing. Data obtained on 14th day postpartum. Numbers beside each point represent number of litters. Vertical bars represent stand. error.

ployed may, in addition to being galactopoetic, also be a limiting factor in milk secretion of many rats since milk yields obtained in GH-treated lactating rats with aid of oxytocin were greater than with oxytocin alone. What is perhaps more significant is that greater reduction in variability of individual yields resulted from the combined treatment. It was interesting to observe that GH-treated rats required 3x more oxytocin to obtain maximum yields of milk than did controls. Increased requirement of oxytocin to evacuate milk in such animals might mean a larger secretory area, thus providing more loci for oxytocin action, or an increased threshhold for myoepithelial contraction. Research in progress may clarify this point.

Summary. (1) We have investigated the effect of growth hormone (GH) and oxytocin alone, and in combination, upon lactation in the rat. Amount of milk obtained by a litter of 6 during 30 minutes nursing on day 14 postpartum, expressed as percent litter body weight, was used as the index of response. (2) Control yields averaged 3.8% with normal distribution. Removal of milk with aid of .1 USP unit/kg oxytocin i.v. resulted in 50% higher yield with more uniformity among individual values. (3) GH injected subc., from days 7-13 at dose of 1 mg/rat/day evoked a 41.1% increase in milk yield. Yields obtained in GH-treated lactating rats with aid of oxytocin were greater and more uniform than with GH or oxytocin alone. The significance of this is discussed. (4) GH had no effect upon pituitary, thyroid or adrenal weight/100 g body weight. GH caused a significant increase in percent weight gain of mothers but not their litters. Litter growth rate as an index of lactation in the rat is discussed.

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Thyroid Hormone and Lactation in the Rat.* (24559)

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Maximum lactation presumably occurs only when each hormone involved in secretion and evacuation of milk is present in adequate quantities. Similarly, any hormone, if present in inadequate amounts, may limit intensity of response though mammary gland growth and other hormone levels may be adequate. This has been shown clearly with regard to oxytocin in which administration of adequate levels to lactating rats resulted in significantly increased milk yields. When combined with growth hormone (GH), a further increase occurred with marked reduction in variability of response among members of the population(1). These data suggested GH also may be a limiting factor in milk secretion in this species. Extensive research, conducted mainly with large animals, has demonstrated that intensity of milk secretion is controlled to some extent by thyroid hormones and that a mild hyperthyroidism is generally beneficial to milk production(2). As it has been observed that milk yield(1,3) and thyroid hormone output(4) varied considerably among individual lactating rats, we have attempted in the present investigation to determine what effect administration of thyroxine alone, and in combination with GH and oxytocin, have upon milk yield. In this way the extent to which thyroid hormones limit intensity of milk secretion in lactating rats was estimated.

Materials and methods. Ninety primiparous lactating rats of the Sprague-Dawley-Ralfsmeyer strain which had reached growth stasis were housed in individual cages under conditions of uniform temperature (78 ± 1°F) in a room artificially illuminated during daylight hours. They were fed Purina Lab Chow and water *ad libitum*. Shortly after birth each litter was adjusted to 6 young and, whenever possible, were equally distributed as to sex. Each lactating rat was injected subc. daily from days 7-13 postpartum with 3.0 μg/100 g l-thyroxine. Thirty received, in addition, GH‡ subc. at a dose of 1 mg/day over a similar period. On day 14, each litter was isolated from their mother for 10 hours, after which mother and young were reunited and permitted to nurse 30 minutes. A few minutes before nursing 30 lactating rats which were treated with thyroxine and 30 treated with thyroxine plus GH were injected subc. with 1 USP unit oxytocin§ to insure maximum milk withdrawal. Immediately after nursing each litter was removed, weighed, killed by decapitation and stomach contents removed and weighed to the nearest .1 g. Each mother also was killed shortly after nursing and her adrenal and pituitary glands rapidly removed, blotted to remove surface moisture and weighed to the nearest .1 mg. Daily body weights of each mother and her litter were recorded.

Results. Weight of milk, expressed as percent litter body weight, obtained on day 14 postpartum by litters of 30 lactating rats injected from days 7-13 with 3.0 μg/100 g/day l-thyroxine, averaged 5.2%, an increase of 37% over normal average yield of 3.8% (Table I). Injection of 1 USP unit oxytocin before nursing on day 14 resulted in a 19% greater average milk yield (6.2%). That obtained with aid of oxytocin from rats injected with l-thyroxine plus 1 mg GH daily from days 7-13 averaged 6.6% which represents a total increase in milk yield of 73.7% over untreated controls. Progressive reduction in variability among individual values also was obtained with addition of each hormone though in each case some overlap with control values occurred. Increased milk yield resulting from l-thyroxine, alone, and in combina-

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‡ The growth hormone (NIH-BGH-1) is a highly purified bovine preparation distributed by Nat. Inst. Health.

§ Kindly supplied by Armour Lab., Chicago.

TABLE I. Effect of l-Thyroxine Alone, and in Combination with Oxytocin and GH, upon Milk Removal by Litters of 6 Young on Day 14 Postpartum. 30 lactating rats in each group.

Treatment	Avg wt of Milk (g)	Litter (g)	Milk / Litter (%)	Mother (g)	Pituitary (mg/100 g)	Adrenal (mg/100 g)
Control*	6.20	163.6	3.8 ± .29[1]	303.5	3.39[5]	23.41[9]
3 μg/100 g/day l-thyroxine subcut. days 7-13	9.55	182.4	5.2 ± .22[2]	285.9	3.71[6]	
Idem, then 1 USP oxytocin subcut. on day 14	11.00	176.6	6.2 ± .14[3]	301.8	3.81[7]	23.23
Idem + 1 mg GH/day subcut. days 7-13, then 1 USP oxytocin on day 14	11.98	181.4	6.6 ± .13[4]	300.5	3.49[8]	26.31[10]

* Milk yield data from Grosvenor and Turner(1).

Student's "t"	Probability
1-2,3,4	.001
2-3,4	.001
3-4	.06
5-6,7	.001
5-8	.1
9-10	.01

tion with GH were not reflected in significant increases in litter growth as analyzed by percent weight gain from days 6-14 postpartum. Body weights of mothers were similarly unaltered (Table II). Thyroxine caused a significant increase in wet weight of maternal pituitary gland. This did not occur, however, in rats treated with thyroxine plus GH (Table I). Adrenal gland weight was significantly increased in lactating rats injected with thyroxine plus GH but was unaffected by thyroxine alone (Table I).

Discussion. It has been suggested that if less than an optimal rate of thyroxine is secreted by dairy cattle, milk production may be depressed below the potential capacity of the animal. By increasing the level of circulating thyroid hormone in such animals, intensity of milk secretion may be increased(2). However, as thyroid hormones are general metabolic stimulants, it is important that levels are administered which stimulate milk secretion maximally without causing undue stress to the animal. In the present investigation daily administration of thyroxine at a level equal to the highest thyroxine secretion rate (TSR) (3.0 μg/100 g) previously observed in our rats during mid lactation(4) did not adversely affect mother body weight indicating that the level employed did not produce severe hyperthyroidism, yet evoked a 37% increase in milk yield. In a previous

TABLE II. Effect of l-Thyroxine Alone, and in Combination with GH, upon Body Weight Gain of Lactating Rats and Their Litters from Days 6-14 Postpartum. Litter weights at day 14 corrected for weight of milk obtained during 30 min. nursing.

Treatment	No. of rats	Avg wt (g) of Litter Day 6	Litter Day 14	Litter Gain	Litter % gain	Mother Day 6	Mother Day 14	Mother Gain	Mother % gain
Control	30	72.0	157.4	84.6	117.5 ± 1.2[1]	297.1	303.5	6.4	2.2 ± .42[4]
3 μg/100 g/day l-thyroxine subcut. days 7-13	30	74.4	165.6	91.2	123.6 ± 1.2[2]	280.1	285.9	5.8	2.1 ± .41[5]
Idem + 1 mg/GH /day subcut. days 7-13	30	77.3	171.1	93.8	122.1 ± 1.1[3]	295.4	300.5	5.1	1.7 ± .52[6]

Student's "t"	Probability
1-2,3	.2
4-5,6	.5

study 1 mg GH/day for same period of time evoked a similar increase in milk yield and when combined with oxytocin greater and more uniform milk yields were obtained than with GH or oxytocin alone. This suggested GH and oxytocin were limiting factors in milk secretion intensity in lactating rats(1). The combination of GH, oxytocin and thyroxine employed in the present investigation elicited still greater average milk yield with pronounced uniformity among individual values. These data suggest suboptimal thyroxine secretion may be limiting intensity of milk secretion in many lactating rats. However, increase in milk secretion as a result of thyroxine administration was not reflected in significant increase in litter weight as reported by Desclin(5). It has been questioned whether, in intact healthy rats, more intense milk secretion can produce litter growth in excess of the inherent capacity of the species (1). Maternal adrenal gland weight per 100 g was not affected by thyroxine, or, in a previous study by GH(1). A combination of 2 hormones, however, produced a significant increase. The observation that thyroxine nullified the GH-induced increase in body weight previously reported for lactating rats(1) suggests heightened glucocorticoid secretion may occur in the enlarged adrenals. Similar effects of thyroxine and GH upon adrenal function have been reported in non-lactating rats(6). Wet weight of the maternal pituitary was increased significantly as a result of thyroxine treatment presumably due to blockage of thyrotropin discharge and subsequent accumulation of the hormone. Combined treatment of thyroxine with GH resulted in no increase over untreated controls. Experiments conducted after those described in the present study demonstrated GH caused significant elevations in TSR and rate of thyroidal I^{131} release in lactating rats(7). Thus the thyrotropin-blocking dose of 3.0 μg/100 g l-thyroxine for normal lactating rats employed in the present investigation might not be sufficient, at least in some instances, to block release of the hormone in animals treated with GH. The possibility also exists that elevation of milk yield of the GH-thyroxine-oxytocin treated group in the present study, over those treated with thyroxine and oxytocin may partially be due to elevated thyroid hormone output in excess of that exogenously administered.

Summary. We have investigated effects of l-thyroxine alone, and in combination with oxytocin and growth hormone (GH) upon lactation in the rat. Amount of milk obtained by a litter of 6 during 30 minutes nursing on day 14 postpartum, expressed as percent litter body weight, was used as the index of response.

1. 3.0 μg/100 g/day l-thyroxine, a level equal to the highest daily thyroid secretion rate previously observed, injected daily from days 7-13, increased average milk yield 37%. That obtained with aid of oxytocin, to insure maximum milk removal, in rats injected for 7 days with l-thyroxine alone, or with 1 mg GH/day, was increased 63.2 and 73.7%, respectively. With addition of each hormone there resulted also a progressive reduction in variation among individual milk yields. It is suggested suboptimal thyroid hormone secretion may limit intensity of milk secretion in many lactating rats. 2. Increased milk secretion, obtained with l-thyroxine with or without GH was not reflected in significant increases in litter growth as analyzed by percent weight gain during period of treatment. Thyroxine had no adverse effect on maternal body weight. Increase in maternal weight due to GH was offset by simultaneous treatment with thyroxine. 3. Thyroxine induced a significant increase in maternal pituitary gland weight which was offset when administered with GH. Adrenal gland weight was significantly increased in lactating rats injected with l-thyroxine plus GH but was unaffected by l-thyroxine alone.

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Thiocyanate Formation by Extracts of *Escherichia coli* and of Liver.* (24560)

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Previous work(1,2,3) has demonstrated the presence of a desulfurase enzyme system in *E. coli* which cleaves β-mercaptopyruvate to free sulfur and pyruvate at pH 7.4. This system appears to be similar to that found in liver although comparison of the properties of the bacterial and mammalian enzymes has not been reported. The present work shows that *E. coli* extracts incubated with β-mercaptopyruvate at pH 8.0 or above do not release free sulfur but transfer it to an acceptor in the environment in a manner analogous to the liver system(4). When cyanide is present, the transferred sulfur readily reacts to produce thiocyanate.

Materials and methods. Escherichia coli (wild type) was grown in the liquid medium of Monod and Williams(5) with addition of 1 g of yeast extract per liter. Ten liters of this medium were inoculated with 100 ml of a 2-day culture and the mixture was incubated at room temperature with vigorous aeration. After 36 hours the cells were collected in a Sharples centrifuge, washed once with distilled water and lyophilized.

Bacterial enzyme preparation. Two g of dried cells and 20 g of alumina were ground in a ball mill for 4 hours at room temperature. One hundred ml 0.01 M K_2HPO_4 were then added and grinding continued an additional 30 minutes. The creamy mixture was centrifuged at 3000 x g to remove the alumina and larger particles and then at 20,000 x g for 1 hour. The supernatant liquor was diluted to 100 ml with distilled water and 5 ml of M $MnCl_2$ were added to remove nucleic acids. The resulting precipitate was removed by centrifugation; excess Mn++ was removed by passing the supernatant through a column of Dowex 50 cation exchange resin, NH_4+ form. This preparation was without thiosulfate transsulfurase activity.

Rat liver preparation. A lyophilized enzyme preparation, made from rat liver acetone powder by the method of Meister *et al.* (1), was purified as described by Kun(6). This material exhibited both thiosulfate and β-mercaptopyruvate transsulfurase activities. *Ammonium β-mercaptopyruvate.* This substrate was prepared by the method of Parrod as modified by Kun(7). *Pyruvate assay.* Pyruvic acid, enzymatically produced from β-mercaptopyruvate, was determined by the method of Friedmann and Haugen(8). *Standard enzyme assay.* The enzyme (0.3 ml) was mixed with 0.5 ml of substrate (84 μmoles) and one ml of 0.2 M "Bis"‡ buffer was added to give a final pH of 9.1. This mixture was incubated for 15 minutes at 37°. Two-tenths ml of KCN solution (28 μmoles) were added and incubation continued for another 15 minutes. Enzyme action was stopped with 0.5 ml of 40% formaldehyde and the volume was made up to 5 ml with water. One ml of Goldstein's reagent(9) was added and the color produced was determined photometrically. The substrate for determination of thiosulfate transsulfurase was $Na_2S_2O_3$ and for β-mercaptopyruvate transsulfurase was NH_4 β-mercaptopyruvate.

Results. The *E. coli* enzyme system was found to be relatively stable at 100° for 5

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‡ Bis = bis(hydroxymethyl)aminomethane or 2-amino-2-methyl-1,3 propanediol.

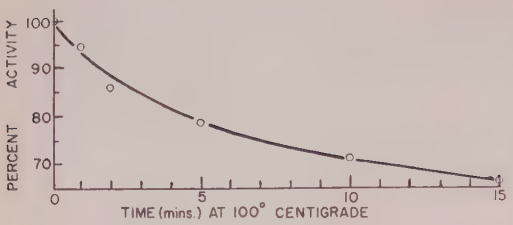

FIG. 1. Effect of heating on *E. coli* enzyme.

minutes (Fig. 1); these conditions completely inactivated the transsulfurases of rat liver. Mild acid hydrolysis of the bacterial preparation also completely destroyed enzyme activity.

Table I shows the bacterial enzyme, unlike

TABLE I. Effect of Sulfhydryl Reagents and Cyanide on β-Mercaptopyruvate Transsulfurase Activity.

Inhibitor (5×10^{-3} M)	% inhibition *E. coli*	% inhibition Rat liver
None	0	0
Iodosobenzoate	13	31
Iodoacetamide	15	24
p-Mercurichlorobenzoate	13	83
Cyanide	28	0

liver β-mercaptopyruvate transsulfurase, to be somewhat insensitive to inactivation by sulfhydryl reagents. In keeping with these observations, the rat liver enzyme was inactivated by air oxidation in presence of copper at pH 6.8 (Table II). These activities were restored completely, or in part, by addition of β-mercaptoethanol which reduces disulfide linkages to sulfhydryl. The enzyme substrate, β-mercaptopyruvate, was not as effective. *E. coli* transsulfurase was not affected by air oxidation.

TABLE II. Effect of Cu^{2+} and Air on β-Mercaptopyruvate Transsulfurase Activity.

Treatment	Addition after treatment	% inhibition Rat liver*	% inhibition *E. coli*†
none	none	0	0
	β-MP‡	29	0
	-ME§	8	3
Cu^{2+}	none	16	2
	β-MP	37	0
	-ME	8	7
Cu^{2+} and air‖	none	75	7
	β-MP	87	2
	-ME	59	3

* Cu^{2+} conc. was 5×10^{-5} M.
† *Idem* 3.3×10^{-5} M.
‡ β-Mercaptopyruvate, 42 μmoles.
§ β-Mercaptoethanol, 84 " .
‖ Aeration was accomplished by blowing a slow stream of water-saturated air through solution for 5 min.

Iodine in the acid pH range oxidizes the sulfhydryl group to disulfide. Table III shows that rat liver β-mercaptopyruvate transsulfurase is inactivated by treatment with iodine at pH 4.5 but is less sensitive than is the accompanying thiosulfate transsulfurase. The bacterial enzyme was not tested since it becomes inactive at acid pH. Iodination at pH 7.4 has the greatest inactivating effect upon the bacterial enzyme. This enzyme system, in contrast to the 2 liver transsulfurases, thus appears to be independent of sulfhydryl group function.

Bacterial transsulfurase is sensitive to cyan-

TABLE III. Effect of Iodine on Transsulfurase Activities.

	% inhibition						
	Rat liver*						*E. coli*
	Thiosulfate‡		Thiosulfate + β-mercaptoethanol		β-Mercapto-pyruvate§		β-Mercapto-pyruvate
μmoles I_2†	pH 4.5	pH 7.4	pH 4.5	pH 7.4	pH 4.5	pH 7.4	pH 7.4
0	0	0	0	0	0	7	0
.1	0	0	0	0	0	3	0
.2	8	0	0	0	1	12	3
.3	91	6	4	2	6	12	23
.4	99	10	27	0	25	46	70
.5	100	6	83	0	31	32	84
.6	100	76	100	0			86

* Protein content of enzyme solution 1.8 mg/ml.
† After treatment with iodine, enzyme activities were determined by standard assay.
‡ Substrate for "rhodanese" activity.
§ Addition was made in 2 equal parts, the second immediately before incubation to reverse oxidation.

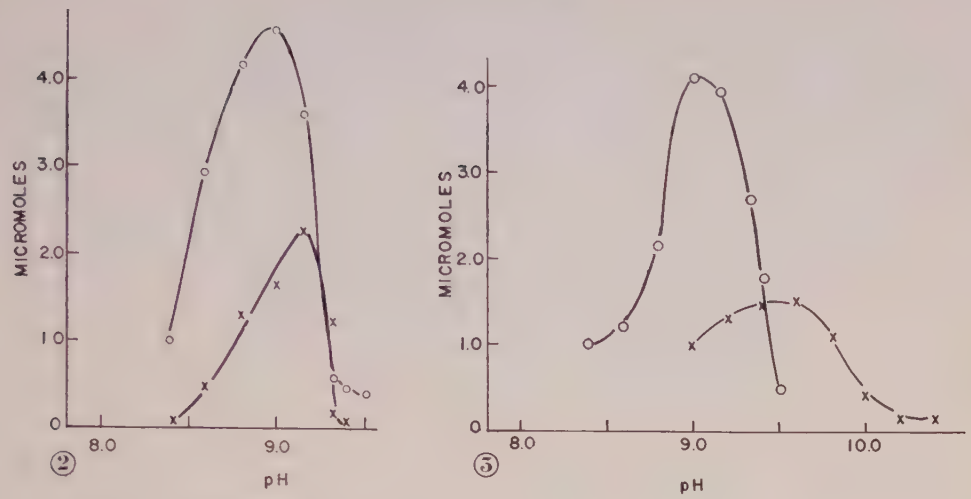

FIG. 2. pH optima of rat liver enzyme. ○——○ = pyruvate, ×——× = KSCN.
FIG. 3. pH optima of *E. coli* enzyme. ○——○ = pyruvate, ×——× = KSCN.

ide, resembling in this aspect thiosulfate transsulfurase(10). Similar inhibition by cyanide has been related by Sorbo(10) to breaking of essential disulfide bonds in thiosulfate transsulfurase.

The bacterial and liver β-mercaptopyruvate transsulfurases evidently catalyze the same reaction, namely conversion of β-mercaptopyruvate to pyruvate and an unidentified reactive form of sulfur. Formation of thiocyanate from β-mercaptopyruvate can be visualized as a 2-step process. The first step consists of conversion of β-mercaptopyruvate to pyruvate accompanied by a transfer of sulfur to the environment. In the absence of an acceptor, free sulfur separates(1,2). If the environment contains a component reactive with sulfur, a valence change may occur, or elemental sulfur may be fixed in compounds such as polysulfides. The second step in thiocyanate formation is not so clearly defined but is the slower reaction. If elemental sulfur is fixed in the polysulfide form, it can readily react with cyanide; free sulfur in the solid phase produces no thiocyanate within the time period of the standard assay procedure. The pH optimum for the first of the above steps was determined for the rat liver enzyme (Fig. 2) and for the bacterial enzyme (Fig. 3). Also shown in the figures is the pH optimum of the 2 steps combined.

The reaction of the second step has not been established to be enzyme-catalyzed. It is possible that an enzyme-sulfur complex exists since the pH optimum for thiocyanate formation in the presence of cyanide is 9.6 for the *E. coli* system and 9.1 for the liver system. Alternatively, polysulfide formation with different types of sulfhydryl groups on compounds in the environment may account for the differences in optimum pH of the 2 systems.

Summary. The properties of enzyme systems of *E. coli* and of rat liver, which degrade β-mercaptopyruvate, have been compared. The bacterial system was much more stable to heat and less susceptible to oxidation. These characteristics have been related to lack of dependence upon sulfhydryl group. At the pH optimum for pyruvate formation, the enzyme activity may be characterized as a transsulfurase rather than a desulfurase. When cyanide is the sulfur acceptor, thiocyanate formation occurs. The pH optimum for *E. coli* system is 9.6 and for rat liver system 9.1.

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Enhancement of Hematopoietic Action of Hydrocortisone by Cobalt in Adrenalectomized Rats. (24561)

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We have recently reported that adrenalectomy interferes with the hematologic response to cobalt in the rat although rats subjected to a sham operation respond to cobalt as readily as normal rats(1). Further analysis of this effect seemed to require experiments in which adrenalectomized rats were treated with both cobalt and adrenal hormones. Several reports indicating that adrenocortical hormones stimulate erythropoiesis in the bone marrow of the adrenalectomized and intact rat(2-5) and induce an elevation in total circulating red cell volume in the normal rat(6) suggested that the hormones of the adrenal cortex should be investigated in this way. The present report describes the results of concurrent administration of cobalt and hydrocortisone to adrenalectomized rats in which it was found that these substances together stimulate erythropoiesis considerably more effectively than hydrocortisone alone.

Materials and methods. Male albino rats of the Wistar (Purdue) strain were studied in 2 experiments: (I), 8 control rats, 8 adrenalectomized rats receiving 2 mg hydrocortisone per kg daily and 8 adrenalectomized rats receiving a combination of 2 mg hydrocortisone and 2.5 mg elemental cobalt per kg daily for a period of 28 days after operation. (II) 8 control rats, 8 adrenalectomized rats receiving 1 mg hydrocortisone per kg daily, and 8 adrenalectomized rats receiving a combination of 1 mg hydrocortisone and 2.5 mg elemental cobalt per kg daily for a period of 28 days after operation. Erythrocyte counts, hemoglobin and hematocrit determinations were made at 10, 20 and 28 days. The hydrocortisone and cobalt injections were started on the day following the operation. Total red cell volumes were determined at 28 days. Cobalt chloride† was given as an intraperitoneal injection daily at a dosage of 2.5 mg elemental cobalt (10 mg $CoCl_2 \cdot 6H_2O$) per kg of body weight. The hydrocortisone acetate‡ was suspended in 0.9% NaCl solution and injected subcutaneously daily at various sites under the skin of the back. Blood was obtained for erythrocyte, hemoglobin and hematocrit determination by anesthetizing the rat with ether and clipping the tail. Erythrocyte counts were done with U.S. Certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Van Allen hematocrit tubes (1.6% aqueous sodium oxalate as diluent) which were spun for 30 minutes at 3,000 rpm with a radius of 17 cm. Hemoglobin determinations were made on the Klett-Summerson colorimeter by the acid hematin method(7). Body weights were determined at the time of the hematologic studies. Red cell volume was

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†Analytical grade $CoCl_2 \cdot 6H_2O$ with maximum limits of impurity for lead of 0.005%.

‡ Hydrocortisone acetate supplied through courtesy of Upjohn Co., Kalamazoo, Mich.

TABLE I. Effects of Combined Injections of Hydrocortisone and Cobalt on Blood Picture of Adrenalectomized Rat.

Day	No. rats	B.W.	RBC	Hemat	HB	MCV	MCH	MCHC	Red cell vol
				EXP. I					
				Nonadrenalectomized—No treatment					
0	8	357.4 ± 23.4	9.5 ± .78	44.4 ± 2.7	16.0 ± 1.3	47.7 ± 1.7	16.8 ± .95	35.8 ± 1.1	
10	8	362.7 ± 22.7	9.5 ± 1.1	44.5 ± 2.1	16.7 ± 1.0	45.7 ± 7.0	17.6 ± 1.3	37.4 ± 1.6	
20	8	389.5 ± 22.5	9.4 ± 1.0	43.1 ± 1.7	15.4 ± 1.3	45.5 ± 4.1	16.6 ± 2.0	35.8 ± 3.2	
28	8	391.0 ± 30.6	8.0 ± 1.8	47.0 ± 1.4	15.0 ± .78	49.3 ± 4.7	18.0 ± 5.0	31.1 ± 1.4	2.68 ±.45
				Adrenalectomized—hydrocortisone—2 mg/kg/day					
0	8	335.4 ± 26.9	8.2 ± .97	43.6 ± 1.6	15.1 ± .81	54.0 ± 8.2	18.7 ± 2.9	34.6 ± 1.5	
10	8	274.3 ± 28.7	13.5 ± 1.2	47.3 ± 3.3	17.2 ± 1.9	35.3 ± 2.6	13.4 ± .60	38.4 ± 2.2	
20	7	281.0 ± 33.6	11.4 ± 1.4	45.5 ± 3.4	17.3 ± 1.7	39.4 ± 5.3	15.3 ± 1.2	38.5 ± 1.9	
28	5	258.0 ± 36.7	11.4 ± 2.0	48.0 ± 5.5	16.6 ± 1.7	42.5 ± 4.2	14.8 ± 2.1	34.7 ± 2.6	2.40 ±.46
				Adrenalectomized—cobalt—hydrocortisone—2 mg/kg/day					
0	8	325.4 ± 36.5	9.5 ± 1.1	44.1 ± 2.8	16.5 ± 1.2	46.9 ± 6.5	17.7 ± 2.5	37.5 ± 1.4	
10	8	270.0 ± 24.1	11.4 ± 1.4	50.3 ± 3.8	19.2 ± .94	44.4 ± 4.4	17.0 ± 2.0	38.3 ± 2.9	
20	7	276.0 ± 28.0	13.5 ± 1.4	55.0 ± 4.6	19.6 ± 2.4	41.1 ± 6.4	14.6 ± 1.8	35.6 ± 3.5	
28	5	223.0 ± 25.3	14.7 ± 1.8	65.6 ± 3.8	19.3 ± 1.7	45.0 ± 4.5	13.2 ± 1.4	29.4 ± 1.4	9.06±1.08
				EXP. II					
				Nonadrenalectomized—No treatment					
0	8	320.6 ± 31.6	8.7 ± .87	41.2 ± 2.0	14.6 ± .60	47.5 ± 5.4	16.9 ± 1.5	35.4 ± 1.3	
10	8	340.6 ± 32.5	9.6 ± 1.3	43.2 ± 1.0	14.7 ± .45	45.7 ± 6.9	15.6 ± 2.3	34.4 ± 1.6	
20	8	357.8 ± 28.4	9.2 ± .96	41.8 ± 2.4	14.7 ± .80	45.5 ± 4.0	16.2 ± 1.9	35.3 ± 1.2	
28	8	379.0 ± 29.8	9.6 ± 1.1	47.0 ± 2.0	15.0 ± .96	49.3 ± 4.8	15.8 ± 3.5	32.0 ± 2.0	2.61 ±.10
				Adrenalectomized—hydrocortisone—1 mg/kg/day					
0	8	321.4 ± 20.9	8.4 ± 1.5	41.8 ± 2.2	14.3 ± .93	50.6 ± 8.9	17.2 ± 2.7	34.0 ± 2.3	
10	8	282.1 ± 24.2	10.0 ± 2.0	40.4 ± 4.5	13.9 ± 2.0	41.6 ± 8.6	14.1 ± 3.3	32.8 ± 4.0	
20	8	260.8 ± 15.7	8.8 ± 2.5	42.0 ± 3.5	14.4 ± .98	53.1 ±11.9	16.1 ± 4.0	35.5 ± 1.2	
28	8	252.0 ± 14.6	9.0 ± 1.9	48.0 ± 2.0	13.5 ± .79	52.5 ±10.7	15.7 ± 3.5	28.2 ± 2.2	3.58 ±.28
				Adrenalectomized—cobalt—hydrocortisone—1 mg/kg/day					
0	8	299.3 ± 24.1	9.0 ± 1.7	40.8 ± 1.2	14.4 ± .99	48.7 ± 9.4	16.6 ± 3.7	35.3 ± 1.7	
10	8	274.0 ± 20.5	10.6 ± 1.4	43.7 ± 3.7	14.7 ± 1.1	41.8 ± 5.4	14.2 ± 2.4	33.8 ± 2.2	
20	7	262.0 ± 47.5	11.9 ± 1.6	47.6 ± 5.5	17.3 ± 1.9	40.3 ± 4.5	14.7 ± 1.9	36.4 ± 2.6	
28	5	241.0 ± 51.0	12.5 ± 1.5	60.0 ± 4.9	18.6 ± 2.1	48.9 ± 4.0	15.0 ± 1.9	32.1 ± 3.6	6.66 ±.66

Day = Day treatment; B.W. = Body wt in g; RBC = Red cell count in millions/mm³; Hemat = Hematocrit in %; HB = Hemoglobin in g/100 ml; MCV = Mean corpuscular vol in μ^3; MCH = Mean corpuscular hemoglobin in $\mu\mu g$; MCHC = Mean corpuscular hemoglobin conc. in %; ± = Stand. dev. and red cell vol = ml red cells/100 g B.W.

determined with P^{32} tagged erythrocytes by the method of Hevesy and Zerahn(8) as modified by Berlin *et al.*(9). The tagged cells were injected into a vein, exposed by an incision on the inner aspect of the thigh, and a blood sample removed *via* heart puncture for counting after 10 minutes. The blood was dried on aluminum planchets and counted with an end window Geiger-Mueller counter. Bilateral adrenalectomy was performed under ether anesthesia by a short transverse incision through the skin and lumbar muscles just below the last rib on each side. In order to insure against leaving behind any closely adhering accessory adrenal tissue, the fat surrounding the gland to a distance of ½ to 1 cm was removed on each side. Each animal adrenalectomized was given 10,000 units of Procaine Penicillin intramuscularly at time of operation and on the following day. The adrenalectomized rats were maintained on 0.9% NaCl. At the end of the study, the adrenalectomized animals were examined for evidence of accessory adrenal tissue. P values were determined by Fisher's "t" test.

Results. The data presented in Table I indicate that the anemia of adrenalectomy was prevented, for the most part, by administration of hydrocortisone at a dose of 1 mg/kg/day. After 28 days of treatment with 1 mg hydrocortisone there was an increase of 15% in hematocrit, 7% in rbc count and a 6% de-

crease in hemoglobin. The MCV was markedly decreased at 10 days but was slightly above normal at 20 and 28 days. The red cell volume was 3.58 ml per 100 g body weight.

Hydrocortisone at a dose of 1 mg/kg per day given concurrently with cobalt at a dose of 2.5 mg/kg daily induced a marked elevation in hematocrit (47%), rbc count (39%) and hemoglobin (29%). The red cell volume was 6.66 ml/100 g at 28 days. MCV was decreased at 10 and 20 days but returned to normal at 28 days. The adrenalectomized rats progressively lost weight when treated with 1 mg hydrocortisone with or without cobalt.

The 2 mg/kg dose of hydrocortisone in adrenalectomized rats resulted in 10% increase in hematocrit, 39% increase in rbc count, and 10% increase in hemoglobin at 28 days. However, total red cell volume was only 2.4 ml per 100 g. The combination of 2 mg hydrocortisone and cobalt induced a pronounced rise in hematocrit (49%), rbc count (55%) and hemoglobin (17%). The red cell volume was elevated to the extremely high value of 9.1 ml per 100 g. Adrenalectomized rats treated with either 2 mg hydrocortisone or the combination progressively lost weight, the former decreasing 30% and the latter 46% in 28 days. Autopsies at 28 days revealed no evidence of accessory adrenals in any adrenalectomized animal receiving either hydrocortisone alone or the combinations.

Discussion. The remarkable increases in hematocrit and red cell volume evoked by the combination at 20 and 28 days suggest a synergistic (more than additive) action between cobalt and hydrocortisone. A thorough analysis of this synergism becomes quite involved because (1) adrenalectomy alone produces temporary anemia(1,4); (2) cobalt in the adrenalectomized animal may correct the anemia during the first 20 days, although satisfactory statistical evidence for this is lacking(1); (3) cobalt clearly stimulates the adrenalectomized rat during the second month after adrenalectomy(1); (4) the complete dose-response curve for hydrocortisone in the adrenalectomized rat is not available; (5) loss of weight in certain groups introduces an extraneous variable and (6) the large number of different groups needed makes it quite difficult to carry out a complete experiment in which all variables are studied simultaneously.

Summary. In the rat, hydrocortisone prevented the anemia of adrenalectomy and in one experiment caused a moderate increase in total red cell volume. Hydrocortisone and cobalt injected concurrently in adrenalectomized rats produced a marked elevation in hematocrit, rbc count, hemoglobin and total red cell volume. This joint action of cobalt and hydrocortisone at least equalled the sum of the separate effects.

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"Plasma Insulin Activity" in Human Diabetes During Hypoglycemic Response to Tolbutamide and Indole-3-Acetic Acid. (24562)

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The principal action of tolbutamide (Orinase) is probably direct stimulation of beta cells of the islets of Langerhans to secrete insulin(1). Conversely, Mirsky's pioneering studies with indole-3-acetic acid indicate that the latter's hypoglycemic effect is due to extra-pancreatic inhibition of insulinase activity(2). After administration of either substance, one might expect depression of circulating glucose levels to be paralleled by increased "insulin activity"* in peripheral blood. It was found, however, that the hypoglycemic responses of normal subjects and mild diabetics to both compounds was not accompanied by enhanced plasma insulin activity.

Materials and methods. Clinical material included 5 metabolically normal subjects, 7 elderly diabetic patients whose fasting blood sugars were easily controlled on diet plus daily tolbutamide, and 5 labile or juvenile diabetics who promptly developed severe ketonuria when exogenous insulin was reduced. All patients were males. Mean age of each group was, respectively, 46, 57, and 39 years (Table I). No elderly patient received insulin within a week before beginning the tests. One patient with juvenile diabetes tolerated complete withdrawal of insulin, but the others required periodic small injections of regular insulin daily to prevent development of frank ketoacidosis. No insulin was given later than noon of day preceding each test. All subjects consumed 300 g of carbohydrate and maintenance calories for 3 days prior to testing. On first test day, samples were obtained for blood glucose and for "plasma insulin activity" in the fasting state, and again 1 hour after taking 100 g of glucose orally. The next day similar fasting samples were drawn, the individual given 100 mg/kg of indole-3-acetic acid† in capsules, and specimens for blood glucose obtained hourly for 5 hours. Between 2nd and 5th hours, a second plasma sample was drawn for insulin assay. Two or 3 days later the procedure was repeated, except that the person received 3 g of tolbutamide‡ orally after drawing fasting specimens, and the second sample for insulin assay was obtained between 1st and 4th hours. Blood glucose was determined in duplicate by the Somogyi-Nelson method(3). Vallance-Owen's rat-diaphragm technic(4) was used to determine "insulin activity" of plasma. The latter was expressed as net glucose uptake from plasma, in mg % per 10 mg dried diaphragm, *i.e.,* total glucose uptake from test plasma by hemidiaphragm minus baseline uptake from buffered glucose solution by control hemidiaphragm. Paired fasting and post-treatment plasmas were always assayed simultaneously.

Results. Tolbutamide and indole-3-acetic acid demonstrated quantitatively parallel hypoglycemic effects throughout (Table I), the latter being slight in normal subjects, impressive in elderly diabetics and essentially zero in juvenile diabetics, respectively. Maximal depression of fasting glucose in normal individuals was 22 ± 4% (mean ± S.E.M.) after tolbutamide, and 11 ± 4% after indole-3-acetic acid. Respective values in mild adult diabetics were 44 ± 4% after tolbutamide and 35 ± 6% after indole-3-acetic acid. Negligible blood sugar changes of 1 ± 1% after tolbutamide, and 7 ± 3% after indole-

* "Plasma insulin activity" refers to biologically effective insulin concentration, *i.e.,* total insulin content minus effect of insulin antagonists in undiluted plasma.

† Purchased from Nutritional Biochemicals Corp., Cleveland.

‡ Generously provided by C. J. O'Donovan, Upjohn Co.

TABLE I. Maximal Depression of Blood Glucose Concentration after Oral Administration of 3 g of Tolbutamide or 100 mg/kg of Indole-3-Acetic Acid to Normal Subjects and Diabetic Patients (Mean ± S.E.M.).

Clinical status of patients (No.)	Age	Tolbutamide		Indole-3-acetic acid	
		Fasting blood glucose (mg %)	Max fall from fasting level (%)	Fasting blood glucose (mg %)	Max fall from fasting level (%)
Normal (5)	46 ± 5	82 ± 2	22 ± 4	87 ± 3	11 ± 4
Adult (stable) diabetes (7)	57 ± 4	187 ± 20	44 ± 4	195 ± 16	35 ± 6
Juvenile (labile) diabetes (5)	39 ± 3	390 ± 49	1 ± 1	420 ± 45	7 ± 3

3-acetic acid, were exhibited by juvenile diabetics.

The data on "plasma insulin activity" (Table II) is subdivided as follows:

(1) *Insulin activity in fasting state.* Rat diaphragm incubated in fasting plasma from either normal subjects or elderly diabetics extracted significantly more glucose than did control diaphragm incubated in buffered glucose solution. In contrast, rat diaphragm showed no net uptake of glucose from fasting plasma of uncontrolled juvenile diabetics. In normal subjects the all-inclusive, average net glucose uptake (mean of all 3 groups of fasting values) was 8.9 ± .9 mg %/10 mg dried diaphragm; this increment above baseline uptake was highly significant ($p<.001$). Comparable net fasting uptakes were 7.1 ± 0.7 ($p<.001$) in elderly diabetics, but only 0.6 ± 0.6 ($p<.7$) in the juvenile group.

(2) *Insulin activity after glucose.* Both normal individuals and mild diabetics also showed statistically significant increases in glucose uptake, above fasting values, 1 hour after ingesting glucose. Respective mean rises above fasting levels were 7.4 ± 1.4 mg %/10 mg diaphragm ($p<.001$) in normals, and 4.8 ± 1.1 ($p<.02$) in elderly diabetics. In uncontrolled juvenile diabetics, circulating insulin activity remained undetectable (0.7

TABLE II. "Plasma Insulin Activity," Expressed as Glucose Uptake by Rat Diaphragm from Plasma of Normal and Diabetic Individuals, after Ingestion of Glucose, Tolbutamide and Indole-3-Acetic Acid.

Clinical status of patients	Net glucose uptake,* in mg %/10 mg dried diaphragm (mean ± S.E.M.)			
	Fasting (A)	Post-treatment (B)	Post-treatment increase above fasting (B − A)	p
(G)		Glucose (100 g)		
Normal	6.4 ± .4	13.8 ± 1.6	7.4 ± 1.4	<.001
Adult diabetes	10.1 ± .9	14.9 ± 1.2	4.8 ± 1.1	<.02
Juvenile "	.8 ± .7	1.5 ± 1.6	.7 ± 1.3	<.5
(T)		Tolbutamide (3 g)		
Normal	7.9 ± 1.1	7.7 ± 1.0	− .2 ± .6	<.9
Adult diabetes	6.1 ± .8	6.0 ± 1.3	− .1 ± 1.3	>.9
Juvenile "	1.7 ± 1.0	−1.0 ± 1.2	−2.7 ± 1.5	<.2
(I)		Indole-3-acetic acid (100 mg/kg)		
Normal	12.4 ± 1.7	10.7 ± 1.6	−1.7 ± 1.3	<.5
Adult diabetes	6.3 ± 1.6	7.6 ± 1.8	1.3 ± 1.1	<.6
Juvenile "	− .3 ± 1.2	− .4 ± 1.8	− .1 ± 1.5	>.9
(G + T + I)	"Grand mean" of corresponding fasting uptake values (A)			
Normal	8.9 ± .9			<.001†
Adult diabetes	7.1 ± .7			<.001
Juvenile "	.6 ± .6			<.7

* Net glucose uptake = uptake from plasma minus baseline uptake from buffered glucose solution.

† Significance of net fasting glucose uptake above baseline uptake.

± 1.3 mg %/10 mg diaphragm) following glucose load.

(3) *Insulin activity after tolbutamide and indole-3-acetic acid.* In contrast to increased glucose uptake by rat diaphragm after glucose, normal subjects and mild diabetics showed no enhancement of insulin activity in peripheral blood after either tolbutamide or indole-3-acetic acid. Mean variation from fasting glucose uptake in normals was –0.2 ± 0.6 mg %/10 mg diaphragm after tolbutamide, and –1.7 ± 1.3 after indole-3-acetic acid. Respective values in mild diabetics were –0.1 ± 1.3 after tolbutamide, and 1.3 ±1.1 after indole-3-acetic acid. None of these minor deviations was numerically significant. In the juvenile group, neither substance improved the already negligible fasting glucose uptake, the mean change being –2.7 ± 1.5 mg %/10 mg diaphragm after tolbutamade and –0.1 ± 1.5 after indole-3-acetic acid.

Discussion. The magnitude of average maximal hypoglycemic responses to indole-3-acetic acid in normal subjects (11%) and mild diabetics (35%) agrees closely with respective mean values of 16% and 38% reported by Mirsky(5). Similarly, the demonstration of "insulin activity" in fasting plasma of normal individuals and mild diabetics, its enhancement in each group 1 hour after glucose ingestion, and its undetectability in juvenile diabetics both before and after glucose, all duplicate the findings of Vallance-Owen (4,6).

The closely comparable hypoglycemic effects of indole-3-acetic acid and tolbutamide in normal subjects and diabetics indicate that the tryptophan metabolite, like sulfonylurea, requires insulin-secreting capacity of the patient. Moreover, failure of the marked fall in circulating glucose shown by mild diabetics, to be accompanied by elevated "plasma insulin activity," suggests that insulin-dependent hepatic mechanisms may underlie the acute hypoglycemic response to both substances. Ricketts(7) proposed that only small, "permissive" amounts of insulin are needed to catalyze predominantly intra-hepatic effects of sulfonylureas, since tolbutamide caused marked lowering of blood sugar in totally depancreatized dogs maintained on constant but minimal exogenous insulin dosage. However, Pfeiffer(8) reported increased insulinlike activity in portal vein blood after administering tolbutamide; and Madison(9) found 54% of endoportally injected insulin-I^{131} bound within the liver of fasting subjects during its first transhepatic circulation. These reports imply that tolbutamide does stimulate beta cells to release insulin, which is largely bound to liver cells in post-absorptive individuals. Insulin-binding in turn may necessarily precede the reduced hepatic output of glucose following administration of either insulin itself (10) or tolbutamide(11). Regarding indole-3-acetic acid, and accepting its postulated role as non-competitive inhibitor of insulinase activity, the highest concentratons of insulinase reside in the liver. Decreased destruction of insulin by insulinase would increase net hepatic content of insulin, which might similarly be bound to liver cells and shut off release of glucose by that organ. Administration of either tolbutamide or indole-3-acetic acid, therefore, might augment intra-hepatic insulin activity and cause hypoglycemia without being paralleled by enhanced insulin activity in peripheral blood. Renold likewise did not find increased plasma insulinlike activity during the acute hypoglycemic response of normal subjects to intravenous tolbutamide(12). The foregoing analysis remains compatible with the demonstration of increased peripheral insulin activity after oral glucose loading. Not only may hyperglycemia be a quantitatively stronger insulogenic stimulus *per se* than sulfonylureas, but the liver may bind progressively less insulin as the fasting state yields to that of sustained hyperglycemia with its increased rate of insulin secretion(13).

Finally, although the findings fully support Mirsky's view that indole-3-acetic acid acts essentially as an insulinase-inhibitor, other interpretations have not been eliminated. Indole-3-acetic acid is also a potent plant growth hormone. Other hypoglycemic mechanisms *via* known or postulated effects of animal growth hormone therefore warrant investigation, such as decreased gluconeogenesis dur-

ing protein synthesis or direct stimulation of beta cells to anatomical and functional hypertrophy(14).

Summary. Hypoglycemic responses of normal and diabetic men to tolbutamide and indole-3-acetic acid were similar both qualitatively and quantitatively. These substances, respectively, depressed fasting glucose levels 22% and 11% in normal subjects; 44% and 35% in elderly patients with mild diabetes; and 1% and 7% in uncontrolled juvenile diabetics. Normal individuals and mild diabetics demonstrated significant "insulin activity" in their fasting plasmas, and both groups exhibited sharp increases in insulin activity after ingestion of glucose. Insulin activity was not detectable in blood of juvenile diabetics either before or after glucose. In no individual, however, whether normal or diabetic, was the hypoglycemic response to tolbutamide or indole-3-acetic acid accompanied by increased "plasma insulin activity" in peripheral blood.

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Metabolism of Sulfobromophthalein Sodium (BSP) in Dog and Man.*† (24563)

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Incomplete recovery of sulfobromophthalein sodium (BSP) from bile following intravenous administration has been attributed either to extrahepatic removal(1) or to BSP metabolism by the liver(2). Brauer and his associates(3,4,5) clarified this problem by a variety of technics including column chromatographic analysis with S^{35} labelled dye as tracer. They have shown that BSP is altered by liver and that the products in bile are colored compounds resembling BSP in their absorption spectra, but apparently differing with respect to extinction coefficients. As a result, colorimetric determinations of BSP in bile are affected by an error leading to systematic underestimation of concentration. Recent studies(6,7) indicate that BSP is transferred into bile by a rate-limited transport system and that it accumulates in hepatic cells in some fixed concentration gradient with respect to plasma. Further understanding of these

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‡ John and Mary R. Markle Scholar in Medical Science.

§ Rockefeller Travelling Fellow in Medicine.

functions requires a knowledge of the character and behavior of the metabolites of BSP. We approached this problem by utilizing the technic of paper chromatography on specimens of plasma and bile obtained during intravenous BSP infusion.

Methods. Four cholecystectomized, splenectomized dogs were fitted with a Thomas duodenal fistula apparatus(8), placed so that the Ampulla of Vater could be visualized. After inserting a ureteral catheter directly into the common bile duct, fasting unanesthetized animals were maintained in upright position by a sling. This preparation permitted accurate collection of timed bile samples by aspiration or gravity drainage. Venous blood was obtained from an indwelling polyethylene tubing. In 5 human subjects, 2 post-cholecystectomy, one with active infectious hepatitis and 2 normal, blood was obtained by venipuncture, and in the first 2 of these, bile was collected from a T-tube. Each of 4 dogs and 5 human subjects was given intravenous infusion of BSP by means of constant delivery pump at a rate equal to or exceeding maximal excretory rate or Tm_{BSP}, which was measured (7). After suitable equilibration period appropriate blood and bile specimens were taken and all analyzed as follows for metabolites of BSP: *1. Preparation of samples for chromatography.* (a) *Plasma concentrations* of BSP were determined in Beckman DU spectrophotometer set at 580 mμ after dilution and addition of alkali. BSP was extracted into protein-free solution and concentrated as follows: Two ml of plasma were mixed thoroughly with 0.36 ml of a saturated solution of ammonium sulfate, shaken with 5 ml of ethanol and maintained at room temperature 30 minutes. After centrifuging, the clear supernatant was aspirated and transferred to a clean tube. In 28 determinations 94.3 ± (S.D.) 4.5% of BSP in original plasma sample was recovered. The supernatant was then evaporated to dryness under air stream in water bath at 40 to 50°C. BSP in the residue was extracted into 0.3 ml of ethanol prior to chromatography. (b) *Biliary concentrations* of BSP, measured colorimetrically, ranged 1000 to 1500 mg %. The high concentrations permitted direct analysis, without concentration or extraction. Occasionally high concentrations of various interfering substances necessitated precipitation and extraction of BSP from bile by the method employed for plasma. Repeated ethanol extractions were required in the final step to ensure complete recovery. No significant differences were observed between chromatograms of extracted and unextracted bile samples when the latter were technically adequate. *2. Chromatography.* The samples were applied in duplicate on Whatman #1 filter paper (17 x 21 inches) in successive volumes of 10 λ or less, allowing each to dry before reapplying until approximately .01 to .02 mg of BSP had accrued. Solvent was prepared as follows: To 4 parts of reagent grade n-butanol mixed with one part of glacial acetic acid distilled water was added in excess. The edge of the prepared filter paper was dipped in the solvent in a sealed container and allowed to stand while the solvent ascended 25-30 cm, usually 14-16 hours at 20°C. The paper was then dried and "developed" by spraying each side with 20% sodium carbonate. *3. Quantitative methods.* Chromatograms revealed one or more discrete areas occupied by BSP-like compounds. Each area (spot), assigned a separate R_f, was cut out and the dye eluted from the paper into 5 ml of distilled water plus 0.1 ml of 30% potassium hydroxide. The amount of BSP in each was calculated from the optical density of its eluate minus an appropriate blank, using a curve calibrated with BSP. The sum of optical densities of all components of the chromatogram gave average recovery of 86% of total applied at starting point, in 9 determinations. The percentage of each BSP-like compound in a given sample was expressed in terms of optical density of its eluate to the sum of optical densities of all components. This percentage includes a systematic error depending upon the extent of assumed but as yet undetermined differences between extinction coefficients of BSP and its metabolites in dog and man. Analysis of chromatograms by this method revealed agreement within 5% in duplicate determinations of any one component in 96 instances.

TABLE I. Average R_f Values of BSP-Like Compounds Found in Bile and Plasma during Constant Intravenous Infusions of BSP.

Subject	Sample	Chromatographic components			
		BSP-I	BSP-II	BSP-III	BSP-IV
Dog	Bile	.48 (52)	.38 (51)	.34 (50)	.30 (50)
Man	"	.47 (2)	.33 (2)	.30 (2)	.28 (2)
"	Plasma	.47 (21)	.38 (9)	.32 (17)	.27 (17)

No. in parentheses indicate No. of determinations.

Results. 1. Demonstration of BSP metabolites in bile and plasma. Four compounds whose absorption spectra were identical with BSP were demonstrated chromatographically in all bile samples of 4 dog and 2 human subjects, and in plasma of 5 human subjects. A photograph of a typical chromatographic pattern of dog bile is shown in Fig. 1. Table I shows average R_f values of the various compounds in the chromatograms, referred to in descending order as BSP-I, BSP-II, BSP-III and BSP-IV. (Chromatograms of dog plasma, although showing presence of several BSP compounds, were technically unsatisfactory). These patterns appear to be similar to those described by Krebs and Brauer(5) for bile of sheep, cat, dog, chicken and rat subjected to column chromatographic analysis. Since similarity of pattern does not necessarily imply chemical identity of the metabolites between species, the animal source should be indicated. The major compound (97.3%) in commercially available BSP was chromatographically identical with BSP-I in the bile and plasma samples. In 2 lots of commercial dye tested, 2 impurities with BSP color were identified chromatographically at R_f 0.95 (0.5%) and 0.84 (2.2%), referred to respectively as BSP-a and BSP-b. The chromatographic character of BSP was unaffected by incubation at 37°C for from 3 to 12 hours with plasma, whole blood and bile from dogs and from normal and jaundiced human subjects. It is therefore apparent that formation of BSP-II, III and IV is dependent upon a metabolic process *in vivo*.

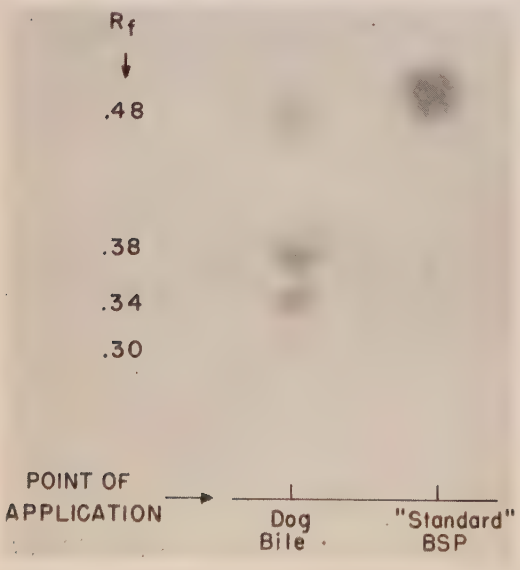

FIG. 1. Photograph of a chromatogram of BSP as excreted in dog bile (left) and as pure BSP (right). The 4 chromatographic components are clearly separated, referred to in descending order as BSP-I, II, III and IV.

2. Properties of chromatographic components. (a) BSP-I was hydrolyzed in 6N hydrochloric acid for 18 hours at 110°C in sealed tubes. Seventy-five per cent of total color was recovered. Chromatography following this treatment revealed that approximately half the dye was unaffected and the remainder consisted of BSP-a and BSP-b. *(b) BSP metabolites.* Insofar as identity of R_f suggests chemical identity, the corresponding compounds from different dogs appear to be the same. This was confirmed by the fact that mixtures of bile samples from different dogs yielded chromatograms identical in pattern. The 2 human samples were not mixed because they were obtained weeks apart. Each of the metabolites in dog bile was chromatographically stable to boiling in air for 3 hours, to addition of 6N HCl, to addition of alkali to pH 12, to drying, and to storage at 4°C. The reduced R_f values of metabolized BSP suggested the possibility of conjugation with one or more polar groups. Hydrolysis with 3N hydrochloric acid for 3 hours at 100°C to split possible glucuronic linkages

TABLE II. Percentage Distribution of BSP and Metabolites in Dog Bile Excreted during an Intravenous Infusion Equal to Tm_{BSP}.

Time, min.	Blood level BSP, mg/100 ml	Bile flow, ml/min.	Total colorimetric BSP output-bile, mg/min.	% of total color in bile of BSP and metabolites			
				I	II	III	IV
22	3.42	.188	2.97	13	54	8	24
39	3.23	.209	2.94	13	53	10	22
50	3.25	.204	2.97	14	55	10	22
68	3.52	.185	2.51	13	55	11	20

did not alter chromatographic mobility of the metabolites. All 3 metabolites in dog bile and BSP-III in human bile in contrast to commercially available BSP or BSP-I in bile, were found to be linked with a ninhydrin reactive substance.

The question of chemical identity of corresponding metabolites of dog and man was not fully answered in this study. However, in contrast to their counterparts in the dog, BSP-II and BSP-IV in human bile were not shown to be linked to a ninhydrin reacting compound. The major constituents of dog and human bile, BSP-II and BSP-III respectively, do not seem to correspond. Present technics permitted isolation of only major fractions in bile and these 2 were analyzed further. BSP-II-dog and BSP-III-man were hydrolyzed with 6N hydrochloric acid in sealed tubes for 18 hours at 110°C. The products of hydrolysis were subjected to 2-dimensional chromatography for amino acids (9,10). Canine BSP-II yielded a single amino acid identified as glycine. Human BSP-III yielded at least 3 amino acids identified as alanine, glycine and glutamic acid. Cysteine and cystine were not detected. Both hydrolysates also contained 2 new derivatives of BSP which moved at R_f .60 and .66 in the n-butanol solvent. These compounds have an absorption spectrum similar to BSP and were not detected in the hydrolysis of BSP-I. Small amounts of BSP-I were also found in the hydrolysates.

3. Physiologic properties of BSP metabolites. A constant biliary excretory rate was observed when infusions of BSP were administered to dogs at rates in excess of Tm_{BSP} (7). Table II shows the proportion of total BSP color attributable to the 4 fractions excreted in bile of dog Cora at various times during constant infusion equal to her previously measured Tm_{BSP}. Table III lists average proportions of the 4 fractions during sustained infusion at or above the excretory maximum on 5 occasions in 4 dogs. The data indicate that BSP and its metabolites are excreted in constant proportions in a given animal and in very nearly the same proportions from dog to dog under conditions of maximal biliary excretory rate of BSP. This did not seem to be the case prior to stabilization at Tm levels. Metabolites could not be detected in plasma of dog or man until at least 30 minutes had elapsed following start of a constant infusion of BSP. The presence of metabolites

TABLE III. Comparison of Mean Percentage Distribution of BSP and Metabolites in Bile of 4 Dogs Obtained during Constant Intravenous Infusion of BSP at Rates Equal to or in Excess of Tm_{BSP}.

Dog	Bile flow, ml/min.	Total colorimetric BSP output-bile, mg/min.	% of total color in bile of BSP and metabolites				No. of determinations
			I	II	III	IV	
Cora	.20	2.8	13	55	10	22	4
Norma	.32	3.8	16	59	16	10	3
"	.31	3.4	11	63	16	11	2
Isolde	.32	3.7	10	53	17	21	4
Tosca	.27	2.8	13	58	23	7	3

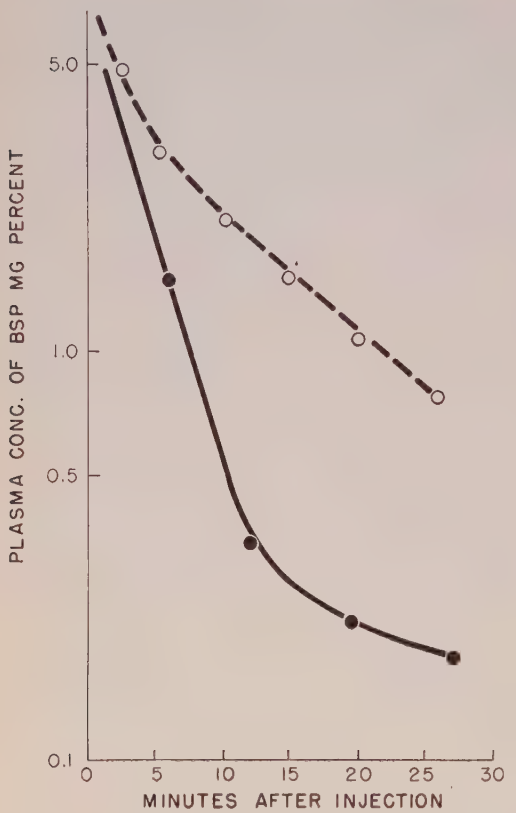

FIG. 2. Comparison of disappearance from plasma following a single intrav. inj. of a mixture of BSP metabolites (○---○) and following administration of same amount of unaltered BSP (●——●) to same dog on different days. Each dose approximately 50 mg.

was most evident in plasma of human subjects with impaired liver function. A solution of metabolites alone was prepared by elution of appropriate areas of a bile chromatogram. This was passed through a Seitz filter and given intravenously to dog Cora. Fig. 2 compares disappearance from plasma of this mixture of BSP-II, III and IV, and a similar amount of unaltered BSP. The normal dog removed unaltered BSP more rapidly than its metabolites following a single intravenous injection. As measured colorimetrically, 97.4% of injected metabolites was recovered in bile in 3 hours, suggesting that the lower recovery of commercial BSP may be due to differences in extinction coefficients(5). Metabolites were not excreted in the same proportion as in the "injectate." Trace amounts of BSP-I, not present in injected material, were found in the last bile samples collected. There also appeared to be some conversion of BSP-II to BSP-III.

Discussion. This study confirms the observations of others that BSP is metabolized by dog and man(5,11). Our evidence supports the conclusion(5,11) that metabolites are not glucuronides. Part of the metabolic change appears to involve conjugation with one or more amino acids. That this is not the only alteration in the molecule is suggested by the following observations: 1) There are significant differences with respect to presence of a ninhydrin reacting moiety in metabolites in dog and human bile although the corresponding metabolites have approximately the same R_f values, 2) 6N acid hydrolysis of 2 metabolites liberate a new BSP derivative, whereas a simple conjugation would be expected to yield unaltered BSP.

Since metabolites are formed by the isolated perfused rat liver(12), and the liver is the major site of BSP removal from plasma, the liver is probably the site of formation of BSP metabolites in dog and man. The presence of these compounds in plasma as well as bile raises the question of whether other tissues may also be capable of metabolism of BSP. Analysis of plasma of hepatectomized animals during BSP infusion will be necessary to answer this question. Our finding that metabolites appear in bile of dogs prior to their detection in plasma is compatible with the inference that the source of plasma components is the liver. The high recovery in bile of intravenously injected metabolites demonstrates that the liver removes these substances efficiently, although at a slower rate than unaltered BSP. Reinjected metabolites also appear to be altered slightly in the process of reexcretion. These findings indicate that removal and excretion of infused BSP from plasma involve the possibility of a number of reactions which proceed simultaneously. The magnitude of these various reactions has yet to be assessed.

At maximal transfer rates quantitatively consistent distribution of BSP metabolites was observed in bile. The significance of this find-

ing is uncertain. It may mean that an excretion maximum exists for each metabolite, but further study is necessary to test this hypothesis. It is apparent from the data that calculation of a true BSP transport maximum is feasible from measurement of total color, percentage distribution of chromatographic fractions and their extinction coefficients.

Summary. During administration of a constant infusion of BSP in the dog and man, 3 chromatographically distinct metabolites were demonstrated in plasma and bile in addition to BSP itself. Part of the metabolic change involves conjugation of BSP or some BSP derivative with one or more amino acids. Some of the physiologic properties of BSP metabolites are described and discussed, particularly with reference to problems of BSP transport from plasma to bile.

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Feather Depigmentation Resulting from Feeding Molybdenum Plus Thiosulfate.* (24564)

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Achromatosis of feathers has been observed in chicks due to deficiency of lysine(1), pantothenic acid(2), and folic acid(3). Achromatosis of the hair of rats resulting from copper deficiency could be corrected by additional dietary calcium pantothenate(4). Although excess dietary molybdenum caused achromatosis of hair in cattle, the literature did not reveal reports of feather depigmentation in chickens due to excess dietary molybdenum. In studies of Miller *et al.*(5) on effect of sulfur containing compounds in alleviation of a molybdenum-induced growth inhibition, it was observed that addition of sodium thiosulfate to a diet containing 1500 ppm molybdenum resulted in feather depigmentation. The data presented here show that feather depigmentation resulting from feeding molybdenum and thiosulfate can be prevented by addition of copper to the diet.

Methods. One-week-old New Hampshire X Barred Plymouth Rock female chicks (10/group) were used except for experiment in which sodium thiosulfate-S^{35} was fed to day-old chicks. The chicks were housed in electrically heated brooders with raised wire floors, and fed the experimental diet for 3 weeks. Feed and water were supplied *ad lib.* Sodium molybdate dihydrate and sodium thiosulfate pentahydrate supplemented the basal diet (Table I). Molybdenum and copper content of basal diet and liver tissues of chicks was determined. The method of Evans *et al.*(6) was used for molybdenum determination and that of Andrus(7) for copper determination. Bone ash was determined by

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TABLE I. Composition of Basal Diet G11.

Ingredients	%
Gr. yellow corn	59.7
Soybean oil meal (44% protein)	35.0
Bone meal	3.0
Salt (4% $MnSO_4$)	.5
Choline chloride supplement (25% choline chloride)	.15
DL-methionine	.20
Vit. B_{12} supplement (10 mg/lb)	.10
A and D feeding oil (2200 A–600 D/g)	.30
	mg/kg
3 Nitro- 4 hydroxophenyl arsonic acid	50
Folic acid	2
Riboflavin	4
Niacin	6
Ca. pantothenate	2
Menadione	2
Dry vit. E (44 IU/g)	600
Chlortetracycline	20

the method of Assn. of Official Agricultural Chemists, 6th Ed.(8). The basal diet contained 3 ppm molybdenum and 13 ppm copper. Radioactivity was determined on duplicate samples using methods of Kulwich *et al.* (10). In one trial sodium thiosulfate-S^{35} (outer sulfur labeled with S^{35}) was added to experimental diets. Each group contained 6 New Hampshire X Barred Plymouth Rock female day-old chicks. Sodium thiosulfate-S^{35} (0.5 mc/kg of diet) was added to diet of groups receiving basal diet, basal diet plus 1.74% sodium thiosulfate · 5 H_2O plus 500 ppm molybdenum. At the end of first week (before feather depigmentation occurred) 2 chicks from each group were sacrificed and S^{35} content of various tissues determined. At end of third week the remaining chicks were sacrificed and sulfur^{35} content of various tissues determined. Autoradiograms were made with wing feathers of groups receiving labeled sodium thiosulfate. Liver samples were hydrolyzed with 3 N HCl for 10 hours. The hydrolysates were fractionated using 0.9 X 100 cm columns of Dowex 50 resin in the hydrogen form(9). After level of radioactivity was determined on fractions obtained from ion exchange columns, the fractions containing radioactivity were concentrated and used for paper chromatography. Methyl ethyl ketone, propionic acid and water (75:25:30), tert-butanol, formic acid and water (75:15:10) and N-butanol, acetic acid and water (40:10:50) were the solvents used to develop the ascending chromatograms.

Results. Supplementation of basal diet with either molybdenum or thiosulfate individually did not cause feather depigmentation. Feather depigmentation caused by feeding molybdenum plus thiosulfate was not prevented by addition of folic acid, calcium pantothenate, or thiamine to the diet (Table II). Addition of lysine to the diet was also ineffective in preventing feather depigmentation caused by feeding molybdenum plus thiosulfate. Feather depigmentation could be prevented by addition of 50 ppm of copper to the diet. When the diet was supplemented with 1.74% sodium thiosulfate · 5 H_2O, as little as

TABLE II. Feather Depigmentation in Chicks.

Group	Treatment	No. of chicks in each group showing feather depigmentation Exp. 1	Exp. 2
1	None	0 (10)†	0 (10)
2	1500 ppm molybdenum	0 (10)	
3	1.74% sodium thiosulfate · 5 H_2O		0 "
4	1500 ppm molybdenum + 1.74% sodium thiosulfate · 5 H_2O	9 (10)	9 "
5	1500 ppm molybdenum + 0.45% sulfur		0 "
6	Same as 4 + folic acid (40 mg/kg diet)		9 "
7	*Idem* 50 ppm copper*		0 "
8	" ca. pantothenate (200 mg/kg) + thiamine HCl (60 mg/kg)		7 (8)
9	" 1% L lysine HCl		8 (9)

* Supplied as cupric carbonate.

† Figure in parenthesis gives No. of chicks remaining in each group when feather depigmentation occurred.

TABLE III. Effect of Varying Level of Molybdenum and Thiosulfate in Diet on Feather Depigmentation of Chicks.

Treatment	No. of chicks in each group showing feather depigmentation
None	0 (10)*
1.74% sodium thiosulfate • 5 H_2O	0 "
250 ppm molybdenum + 1.74% sodium thiosulfate • 5 H_2O	8 (8)
500 *idem*	6 "
1000 "	8 "
1500 "	7 (7)
1500 ppm molybdenum + .35% *idem*	1 (10)
Idem .70% "	3 "
" 1.05% "	3 (9)
" 1.40% "	7 (8)

* Figure in parenthesis gives No. of Chicks remaining in each group when feather depigmentation occurred.

250 ppm molybdenum would cause feather depigmentation (Table III). However, if the level of sodium thiosulfate • 5 H_2O added to diet was less than 1.74%, then addition of 500 to 1500 ppm molybdenum to the diet was required to produce feather depigmentation.

The results of copper and molybdenum determinations on liver tissue are given in Table IV. The chicks having feather depigmentation caused by molybdenum and thiosulfate had a higher copper and molybdenum liver storage than either the basal group or the group supplemented with molybdenum. Feather depigmentation in these experiments is shown in Fig. 1. An autoradiogram of normal and depigmented wing feathers is shown in Fig. 2. Feathers in top row were from chicks receiving thiosulfate. Feathers in bottom row were from chicks receiving molybdenum plus thiosulfate. Areas on feathers lacking pigmentation correspond to the light areas in feathers in bottom row of the autoradiogram. Distribution of S^{35} in tissues of chicks sacrificed at end of third week is shown in Table V. Distribution of S^{35} in tissues of chicks sacrificed at end of first week was similar to distribution of S^{35} at end of third week.

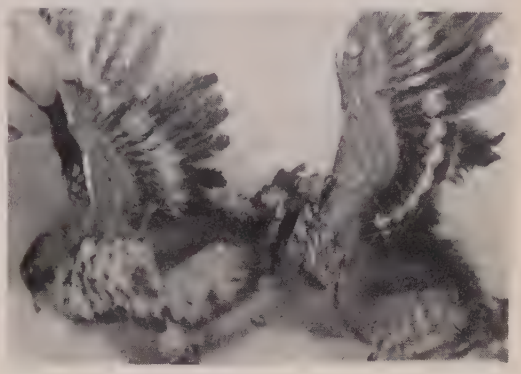

FIG. 1. Feather depigmentation caused by high levels of molybdenum and thiosulfate in the diet.

Discussion. Although feeding molybdenum or thiosulfate will not produce achromatosis of feathers when fed individually, feather depigmentation occurs when molybdenum and thiosulfate are fed jointly. To eliminate the possibility that depigmentation in these experiments was due to a genetic characteristic of crossbred chicks, other colored breeds were fed molybdenum plus thiosulfate. Feather depigmentation was observed in Minorca, New Hampshire, Australorp and Ancona chicks fed molybdenum plus thiosulfate.

Feather depigmentation did not appear until chicks were fed molybdenum plus thiosul-

TABLE IV. Effect of Dietary Molybdenum and Thiosulfate on Molybdenum and Copper Storage in Liver Tissues.

Treatment		Liver tissues (dry wt)*			
		Molybdenum		Copper	
ppm molybdenum	Sodium thiosulfate	Exp. 1	Exp. 3	Exp. 1	Exp. 3
None		9 ± 5	.8	14 ± 2	19 ± 2
1500		25 ± 6		24 ± 12	
	1.74% • 5 H_2O		.7		16 ± 3
250	"		8.0 ± 2		34 ± 17
500	"		9.0 ± 2		30 ± 8.0
1000	"		13.0 ± 3		34 ± 5
1500	"	36 ± 5	17 ± 3	46 ± 10	40 ± 13

* Each value represents avg and stand. dev. for molybdenum and copper determinations made on 5 chicks.

FIG. 2. Autoradiogram of feathers of chicks fed sodium thiosulfate-S^{35}. Top row: Thiosulfate only. Bottom row: Thiosulfate and molybdenum.

fate for one week. Depigmentation was most pronounced in the wings. As experimental period increased, depigmentation was seen in all feathers (Fig. 1). It has been shown that copper is essential for normal pigmentation in mammals. However, when molybdenum and thiosulfate were fed, the livers of chicks showing feather depigmentation had a greater copper content than chicks having normal feather pigmentation. Although chicks showing feather depigmentation had adequate stores of copper in the liver, it would appear that this copper was not available to the chick since addition of copper to the diet prevented feather depigmentation. It has been shown that substances that combine with copper or certain reducing substances like ascorbic acid will prevent tyrosinase from forming melanin from tyrosine(11). When feather depigmentation was first noted with diets containing molybdenum plus thiosulfate, it was suspected that the thiosulfate was either forming a copper complex or inhibiting the tyrosinase by its properties as a reducing agent. However, feeding thiosulfate without molybdenum did not cause feather depigmentation. Furthermore, addition of 1% stannous chloride to the diet containing 1500 ppm molybdenum did not cause feather depigmentation. Addition of 1% ethylenediaminetetraacetic acid (EDTA) to a diet containing 500 ppm molybdenum reduced the growth rate by approximately 25% at 4 weeks of age but did not cause feather depigmentation.

The heart, liver and kidney tissues of chicks receiving molybdenum plus thiosulfate contained more radioactivity than either the basal group or the group receiving supplementary thiosulfate (Table V). Since the group hav-

TABLE V. Distribution of $Sulfur^{35}$ in Chicks after Feeding Sodium Thiosulfate-S^{35} for a 3-Week Period.

	% dose × 10^2/g wet tissue								
	Heart	Liver	Spleen	Kidney	Gall bladder	Skin	Thigh muscle	Blood cells*	† Plasma
Basal diet	11.9	2.9	7.5	2.0	5.8	2.4	5.4	7.9	1.1
" + 1.74% Na thiosulfate • 5 H_2O	2.3	1.1	2.1	.7	1.9	.6	1.3	2.2	.5
Idem + 500 ppm molybdenum	53.8	27.4	4.4	22.0	3.5	1.4	3.9	4.9	.6

* % dose × 10^2/ml packed cell vol. † % dose × 10^2/ml plasma.

ing feather depigmentation had a higher tissue retention of copper, molybdenum and sulfur-S^{35} than the basal or the group receiving thiosulfate, it would appear that feather depigmentation was caused by copper-molybdenum-thiosulfate complex which renders the copper in tissues unavailable to the animal. Although chicks fed molybdenum plus thiosulfate, retained more of the radioactive sulfur than the other 2 groups, conversion of thiosulfate-S^{35} to the organic form was similar in all 3 groups. All of the sulfur^{35} incorporated into organic material was found in the taurine fraction. No radioactivity was found in methionine or cystine fractions. In the 3 solvent systems used for paper chromatography of fractions obtained from the ion exchange columns, the taurine fraction had RF values similar to the RF values obtained with pure taurine. After developing paper chromatograms with ninhydrin, the paper strips were scanned for radioactivity. All radioactivity was found in the spot identified as taurine. Machlin and Pearson(12) reported that the chicken can convert sulfate sulfur^{35} to taurine, methionine and cystine. They reported that most of the sulfur^{35} found in organic form was isolated as taurine, whereas only a small amount was found in methionine or cystine. While it has been shown that taurine is involved in synthesis of taurocholic acid(13), the physiological significance of taurine for any other function is not known.

No differences were noted in hemoglobin values or erythrocyte counts on blood obtained from chicks having normal and abnormal pigmented feathers. Although many chicks fed molybdenum plus thiosulfate had leg deformities, the values for bone ash of this group were similar to bone ash values obtained for the basal group and the group fed thiosulfate. Possibly manganese is also rendered unavailable to the chick when molybdenum plus thiosulfate is added to the diet.

Summary. These experiments show that addition of molybdenum plus thiosulfate will cause feather depigmentation in growing chicks. Supplementation of the diet with vitamins or lysine did not prevent feather depigmentation when the diet contained molybdenum and thiosulfate. No feather depigmentation occurred if copper (50 ppm) was added to the diet. Chicks having feather depigmentation had higher copper, molybdenum and sulfur^{35} content in liver tissues than the basal group or groups receiving supplementary thiosulfate or molybdenum. In all groups fed radioactive sodium thiosulfate, radioactivity was found in the taurine fraction but not in methionine or cystine fraction of liver hydrolysates.

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Decrease of Respiration by Glucose (Crabtree Effect) in Rous Sarcoma of Chorioallantoic Membrane.* (24565)

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Crabtree's observation(1) that addition of glucose to respiring tumor slices produced a reproducible decrease in respiration has been confirmed in Ehrlich ascites tumor(2) and studied in reconstructed systems(3,4). It is also reported to occur in the developing retina but not in adult retina(5). The following report presents data demonstrating this effect of glucose metabolism in a virus-induced tumor.

Materials and methods. Virus. A standard lot of Rous sarcoma virus(6) (CT 669) was obtained from Dr. W. Ray Bryan of National Cancer Inst. and stored in dry ice chest until used. The virus inoculum was 0.2 ml of a 10^{-2} dilution of CT 669 in sterile physiological saline containing 2% inactivated horse serum as a stabilizer of viral infectivity. After thawing a vial of standard preparation of virus, the dilution was made and kept in ice water bath and not held longer than 1 hour from time of thawing of virus preparation until inoculation onto the chorioallantoic membranes. Fertile chicken eggs obtained from a local hatchery were incubated at 39°C for 9 days. False air sacs were made as described by Beveridge and Burnet(7). Volumes of 0.2 ml of virus or diluent as control were inoculated onto the dropped chorioallantoic membrane and the eggs were closed with a patch of Scotch tape. The eggs were further incubated at 39°C, candled daily and dead eggs were discarded. After 9 days' incubation coalescing tumors were harvested from infected eggs and the chorioallantoic membranes were harvested from saline inoculated control eggs after peeling off the remaining shell membrane. *Preparation of tissue for Warburg.* Tumor tissue and normal chorioallantois were excised aseptically from 18-day-old eggs. The tissue was minced finely enough to be drawn into a 1 ml serological pipette, washed 3 times each in 10 to 20 volumes of Hanks' balanced salt solution without glucose (BSS) by centrifuging at 500 rpm for 3 minutes and resuspended as final 20% suspension by volume in BSS. One ml of tissue suspension was added to the reaction chamber of Warburg vessels, of standard size—about 15 ml. The direct method of Warburg was used to determine oxygen uptake of normal chorioallantois and virus-induced chorioallantoic membrane tumors. To 1 ml of tissue suspension in the reaction chamber was added 1 ml of either BSS, BSS containing 0.2% glucose, or BSS containing 0.2% sodium pyruvate. Respiration studies were carried out at 37°C. The dry weights of tissue were determined from contents of the reaction chamber after removal and drying overnight at 80°C on weighed watch glasses and subtraction of the dry weight of 2 ml of the appropriate comparative medium. Tissue per flask averaged about 20 mg dry weight.

Results. Data shown in Table I are from duplicate sets of experiments. Other experiments repeated at intervals of several months have given similar results. Qo_2 values determined at 2 and 4 hours show an observable depression in rate of oxygen uptake of the sarcomatous chorioallantoic tissue when 0.2% glucose is present. With glucose as substrate normal chorioallantoic membrane showed little difference in rate of oxygen uptake from endogenous levels. A pH drop from 7.4 to 6.6 with Rous sarcoma and 6.8 with normal chorioallantois with glucose as substrate was observed at 4 hours. When 0.2% pyruvate was present as substrate there was a rise in Qo_2 values in tumor tissue and normal tissue over the endogenous levels.

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TABLE I. Comparative Rates of Respiration of Normal Chorioallantois and Rous Sarcoma Virus-Induced Chorioallantoic Tumor.

Tissue*	μl O_2/mg dry wt/hr					
	BSS		BSS glucose		BSS pyruvate	
	2 hr	4 hr	2 hr	4 hr	2 hr	4 hr
RSV tumor	3.5	3.6	1.0	1.0	5.5	6.0
	2.3	2.8	.4	.7	2.5	3.0
		pH 7.5		pH 6.6		pH 7.8
Chorioallantoic membrane	2.2	1.9	2.7	1.9	6.5	8.5
	2.5	2.0	2.5	2.5	7.5	7.8
		pH 7.5		pH 6.8		pH 7.8

* All tissue was chorioallantoic membrane from 18-day-old embryonated eggs. BSS: Balanced salt solution; with .2% glucose; with .2% sodium pyruvate. pH, avg at 4 hr.

Discussion. A comparison of Qo_2 values of virus-induced chorioallantoic tumors with normal chorioallantois shows that presence of glucose results in marked depression in respiration of tumor tissue, but not of normal chorioallantois. Aerobic glycolysis as indicated by lowering of pH of medium appeared to be somewhat greater in tumor tissue. Smith and Kun(8) in their studies with Rous sarcoma virus infection of the chorioallantois at 48 hours found increased anaerobic hexose diphosphate dismutation in homogenates as compared with those of normal chorioallantois but their Qo_2 values were 12 and 14 respectively for whole membranes with glucose as substrate. They did not consider this difference as an effect on respiration and they did not give data on endogenous Qo_2. Since very little tumor growth is observable at 48 hours the discrepancies between their results and ours are attributable to differences in age and maturity of the tumors. Our studies compare respiration of minced tumors taken 9 days following inoculation of virus onto the chorioallantois of 9-day-old embryos (total 18 days) with normal chorioallantois taken from eggs of the same age injected with saline. Depression of respiration by glucose was also found in 7-day tumors. Smith and Kun studied the effect of myxoma virus on respiration of chorioallantoic tissue for 12 to 288 hours after infection and observed very little difference between normal and infected whole membranes in the presence of glucose. However, the Qo_2 values of the infected tissues tended to be slightly lower than the controls between 48 and 288 hours.

Usually there is a rise in Qo_2 values over the endogenous rate when 0.2% pyruvate is present with normal or tumor tissue, however, repeated determinations have occasionally failed to show an increase. These variations with pyruvate as substrate may possibly be explained by differences in the stored reserves of endogenous substrate of tissues and the effect of maintaining the tissues in BSS until setting up a determination. Tissues are kept in BSS 1 to 2 hours before a Warburg determination.

It should be noted that with Ehrlich ascites cells(9) concentrations of glucose much lower than those used in our experiments do not inhibit respiration but maintain it above the endogenous level for some time, although higher concentrations are depressive. We have not yet tested the effect of various glucose concentrations with Rous sarcoma.

Summary. Qo_2 values of Rous sarcoma virus-induced chorioallantoic tumors show a depression in oxygen uptake when glucose at 0.1% final concentration is added as substrate. Respiration of normal chorioallantoic membrane was not depressed by addition of glucose. The reaction of the medium with glucose dropped from pH 7.4 to 6.8 with normal tissue and to 6.6 with tumor tissue during 4 hours. Pyruvate as substrate gave increased oxygen uptake with both tumor tissue and normal tissue. These data demonstrate in a virus-induced tumor a metabolic alteration associated with glucose metabolism comparable to that observed in tumors not demonstrated to be of viral etiology.

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Nutritive Substances and Reconstitution in Tubularia. (24566)

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Experiments of Barth(1) and of Rose and Rose(9) showed that substances circulate in the tubularian coelenteron which influence hydranth reconstitution. Barth(1,2,3) postulated that these substances are nutritive and that dominance of distal over proximal end is a result of the competition of the two for a postulated substance which he calls "S." His data can be interpreted either as the result of stimulatory or of inhibitory substances. The latter possibility was suggested by the experiments of Goldin(4,5) in which inhibition was produced by increasing hydrogen ions in the surrounding medium. In addition, Miller(6, 7,8) found that a decrease in pH of similar magnitude as found inhibitory by Goldin, occurred in the coelenteric fluid of stems which failed to reconstitute when placed in glass tubes. The experiments described below were designed to test the effects of reducing the amount of possible nutritive substances which might be available to the distal end of the stem during reconstitution.*

Material and methods. For the first experiments, Tubularia were collected from the Oceanographic Dock at Woods Hole. For later series they came from the U.S. Engineers Dock at Sagamore, Mass. The apparatus for maintaining a constant flow of seawater through the stems consisted of a battery jar 27 cm diameter, with wooden cover containing 21 holes in 2 concentric rings (Fig. 1). Filtered seawater was introduced through a glass tube inserted in the middle hole. In the other 20 holes were placed eye-droppers with tips drawn out to a diameter of less than ½ mm. These were inserted into proximal ends of 10 mm long segments of Tubularia stems. To insure uniformity the distal end of each stem was cut transversely 5 mm below the hydranth and to prevent possible mistakes in orientation the proximal cut was oblique (Fig. 2). After insertion each stem was checked to make certain that the eye-dropper was not occluded by tissue and that a free flow of seawater took place. Controls were given no further treatment and the eye-droppers gradually filled with seawater to the same level as that in the jar. A glass tube with 11 nipples and bent to fit the battery jar was placed on the cover. One nipple was connected through a water trap to an aspirator. The other 10 were connected to short lengths of glass tubing inserted into the eye-droppers as far as the constriction (Fig. 1). Flow of aspirated air was controlled by screw clamps (not shown in Fig.). By this means a difference of about 4 cm in the water level inside and outside of eye-droppers of experimental stems was maintained (Fig. 2). This produced a gentle flow of seawater which, usually, was sufficient to prevent closure of the ends of the stems but was not strong enough to flush the coenosarc out of the perisarc. Patency of the experimental stems was determined at intervals by stopping the aspirator for one-half hour and observing the rise of water in the eye-droppers. Stems which

* Biol. Bull., 1950, v99, 361.

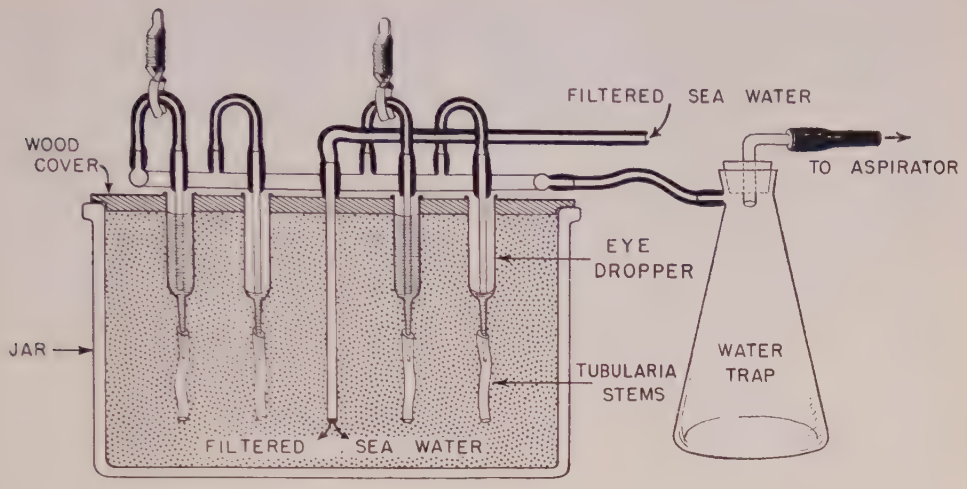

FIG. 1.

failed to show a normal rise were discarded. Filtered seawater was provided at a rate 1 to 1½ liters/minute by a rapid filter constructed from a 2 gallon carboy with a nipple just above the bottom. In this was placed first, 4½ inches of thoroughly washed sand and on top, 2 inches of glass wool to prevent loss of

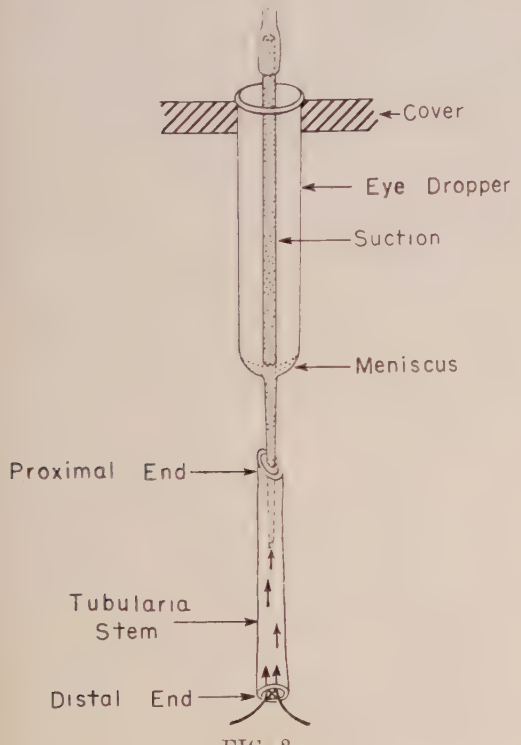

FIG. 2.

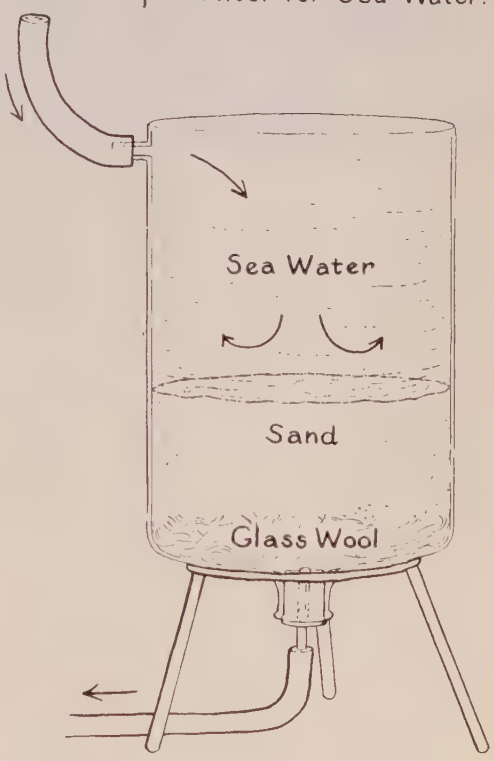

FIG. 3.

sand when the carboy was inverted. The mouth was closed by a one-hole stopper which in turn was wired to the neck of the carboy. Pressure tubing was used throughout and connections wrapped with wire for security. The

TABLE I. Reconstitution of Stems in Running Filtered Seawater.

	No.	No. discarded		No. used in calculations	Reconstitution		
		Plugged	Coenosarc lost		Primordia	Emerged	%
Exp. I							
Control	10	0	0	10	7	7	70
Exp.	10	1	2	7	7	7	100
Exp. II							
Control	10	0	0	10	8	8	80
Exp.	10	2	2	6	6	6	100
Totals							
Control	20	0	0	20	15	15	75
Exp.	20	3	4	13	13	13	100

filter was inverted, connected with the experimental jar and completely filled with seawater through the upper opening. Then tubing connected with the seawater line and completely filled was attached to the carboy. Finally the spigot of the seawater line was opened slowly until the desired rate of flow was attained. When, after 3 or 4 days of continuous use, debris on surface of the sand reduced the rate of flow, it was restored by tilting the carboy sufficiently to break up the surface film.†

Results. In a preliminary experiment no provision was made for circulating the seawater in the battery jar. Neither the experimental nor the control stems reconstituted under these conditions.

In the second experiment circulation was provided but the water was unfiltered. Reconstitution was very slow in all stems and contaminating growth of ciliates and other microscopic organisms developed on the stems. One control and 3 experimental stems lost their coenosarcs either through the action of parasites or (in the case of the experimental stems) because of excessvie flow through the stems. A fourth experimental stem was discarded because it became plugged. Only 4 of the remaining 9 control stems reconstituted (44%). Their hydranths were very small and required 6 days to emerge as contrasted with the usual time of 48 hours. Three of the 6 experimental stems (50%) started reconstitution although in only one did the hydranth emerge. It appeared probable that contaminating growth on the other 2 was responsible for bringing reconstitution to a halt at the hydranth primordium stage. Since a slightly higher percentage of experimental stems than of controls began reconstitution, the results suggested that nutrient materials within the stem may not be essential for reconstitution. However, the only conclusion that could be drawn was that seawater flowing through the jar would have to be filtered.

Table I illustrates results of 2 experiments using seawater which had been passed through the rapid filter. Seven stems were discarded from the experimental group, 3 because they became plugged and 4 because of loss of coenosarc. The remaining 13 stems produced hydranths which emerged. Fifteen of 20 control stems (75%) reconstituted. Thus, stems from which all circulating substances were aspirated produced higher percentages of hydranths than did those which were permitted to close off cut ends and thereby retain any substances liberated into the coelenteron. Consequently, it was concluded that there is no substance essential for hydranth reconstitution which circulates within the tubularian coelenteron.

Discussion. It is very difficult to distinguish between results produced by stimulators and those produced by inhibitors of reconstitution. Barth(1,2,3) postulated that the 2 ends of a stem segment differ in their ability to utilize circulating nutritive substances. However, his results can be interpreted equally well by assuming a differential tolerance of circulating inhibitors. The results reported here cannot be reconciled with

† The author wishes to thank Dr. Arthur L. Colwin for suggesting this type of rapid filter.

the concept that cells in the middle of the stem liberate substances which are necessary for or contribtue to reconstitution. The constant flow of seawater into the distal end and out of the proximal would prevent any substances liberated into the coelenteron from reaching the distal end. By contrast however, the higher percentage of reconstitution which was recorded for experimental as contrasted with control stems is consistent with expectations if there were inhibitory substances within the coelenteron. The direction of flow would be as successful in preventing inhibitors from reaching the distal end as it was in siphoning off nutritive materials. Since increased acidity of the coelenteron occurs during reconstitution, and builds up to concentrations which are known to be inhibitory when externally applied, the suggestion is made that removal of such substances was responsible for the increased percentages of reconstituted hydranths in our experiments.

Summary. An apparatus was constructed which produced a constant flow of filtered seawater through the coelenteron of Tubularia stems. This arrangement was designed to prevent substances liberated into the coelenteron from reaching and influencing reconstitution at the distal end. Reconstitution occurred in spite of removal of such substances and was, in fact, superior to that of control series. Accordingly, it was concluded that although circulating inhibitory substances have been demonstrated, there is no evidence that any substances in the coelenteron influence reconstitution in a favorable direction.

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Total Blood, Erythrocyte and Plasma Volumes in Muscular Dystrophic Mice.* (24567)

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The marked decrease in skeletal muscle mass and lowered body weight observed in mice with hereditary muscular dystrophy evoked an inquiry whether or not the circulatory system was in any way altered to adjust to this condition. Since no reports of total blood volume in either mice or man afflicted with muscular dystrophy were found in the literature, experiments to determine total blood, plasma and erythrocyte volumes of muscular dystrophic mice were performed and the results are presented here.

Methods. Dystrophic mice were obtained from Roscoe B. Jackson Memorial Laboratory and after a week of acclimatization were studied at 43-52 days of age. Littermate normals of the same sex were used in all except 3 instances in which controls were obtained from other litters but of the same stock and age. Other normal but older mice of the same stock were also used, some of which had become very obese. Plasma volumes were measured by T-1824 dye dilution technic and hematocrit values were obtained by using Van Allen tubes. These procedures were used as previously described(1) except for the dye volume. The plunger of a 0.25 ml hypodermic syringe was calibrated to deliver 0.05 ml of dye through a 26 gauge, ½ inch needle inserted into exposed jugular vein in mice under light ether anesthesia. Dye injection was ac-

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TABLE I. Total Blood, Erythrocyte and Plasma Volumes in Normal and Muscular Dystrophic Mice.

Group & No.	Body wt, g	Total blood vol	Erythrocyte vol	Plasma vol	Hematocrit, %
		ml/100 g			
Normal mice (9)	27.8 ± 1.1	10.9 ± .3	5.1 ± .3	5.8 ± .2	47.5 ± 1.6
Littermates of dystrophic mice (9)	20.6 ± .6	10.9 ± .4	5.4 ± .2	5.5 ± .3	49.2 ± 1.0
Dystrophic mice (9)	14.6 ± .4	11.0 ± .5	5.4 ± .3	5.6 ± .3	49.4 ± 1.2
Obese normal mice (4)	44.8 ± 2.1	*8.2 ± .7*	*4.0 ± .4*	*4.2 ± .4*	48.7 ± .5

Means and stand. errors presented; italicized values significantly different, P = 0.01 or less, from appropriate controls.

complished with the aid of binocular dissecting microscope and blood samples were taken by heart puncture. Total blood, erythrocyte and plasma volumes are presented as ml/100 g of mouse body weight. The data were analyzed by the small sample *t* test, and mean differences were considered significant if value of P was 0.01 or less.

Results. The results appear in Table I. Two groups (Groups 1 and 2) of normal mice are shown but only those in the second group were of same ages as the dystrophic mice (Group 3). However, mean total blood, erythrocyte and plasma volume/body weight ratios and hematocrit values in Groups 1 and 2 are not significantly different.

The dystrophic mice, although much lighter than their normal controls, were identical to them in hematocrit value and had proportionally as much total blood.

The small group of older obese normal mice (Group 4), when compared with Groups 1 and 2 treated as a statistical unit, had significantly less blood/100 g body weight but did have the same distribution of cells and plasma as all the other mice.

In none of the groups was there any evidence of sex differences in the blood picture and the data were thus combined.

Discussion. The blood picture obtained in normal mice agrees with published data using similar dye methods(2) and those using radioiodinated albumen(3). In spite of a 33% difference in body weight, due in large part to decrease in muscle mass in the dystrophic mice, the quantities of these blood constituents on basis of body weight were proportionally the same as in controls. Furthermore, it seems unlikely that the vascular space in dystrophic muscles themselves is different from that in controls. Approximately 20% of total plasma volume of mice resides in skeletal muscle which constitutes 40% of body weight (3), and if there were a different ratio of plasma volume to muscle in the dystrophic mouse, it would likely be detected in total plasma volume (and total blood volume assuming a normal hematocrit). Such was not the case.

Certain human muscular dystrophies may involve limited groups of muscles, but in the mouse, the atrophic changes are widespread (4) and vascular development is maintained in constant relationship to body weight. Blood volume determination in muscle biopsy samples was reported for dystrophic and normal muscles of man, and although the results were highly variable, the authors concluded that the blood volumes were not different(5).

Although the effects of anesthesia on blood volume determinations are well known(3), it seems unlikely that anesthesia would nullify any basic difference that might be in the parameters considered in these experiments. The decrease in blood volume in older obese mice can be explained by the well known decrease which occurs with age(2) and also in part by the increase in poorly vascularized adipose tissue.

Summary. Plasma volume (dye method) and hematocrit value were measured in normal and muscular dystrophic mice, and from these data total blood and erythrocyte volumes were calculated. These 2 groups of mice possessed almost identical total blood, erythrocyte and plasma volumes per 100 g of body weight, but the muscular dystrophic mice were 33% lighter than their littermate controls.

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Localization of 5-Nucleotidase and Non-specific Alkaline Phosphatase By Starch Gel Electrophoresis. (24568)

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Bone, intestine, and possibly liver produce non-specific alkaline phosphatase. A specific alkaline phosphatase, 5-nucleotidase, is present in normal serum(1). It is elevated in sera of patients with biliary cirrhosis and obstructive jaundice of extra hepatic origin, and normal in those with bone diseases(1). The present work describes the electrophoretic separation of serum 5-nucleotidase and non-specific alkaline phosphatase by the starch gel technic. These experiments are part of an integrated study of the biochemical and kinetic properties of these enzymes.

Methods. All sera were obtained in the fasting state. The study group included 6 normal adults and 3 normal children, and 20 patients with a variety of disease states, particularly those associated with elevation of serum non-specific alkaline phosphatase. In the latter group were 5 patients with Paget's disease, 2 with osteomalacia secondary to malabsorption, 5 with biliary cirrhosis, 3 with Laennec's cirrhosis, 3 with biliary obstruction secondary to choledocholithiasis and carcinoma, and 2 with sarcoidosis. Non-specific alkaline phosphatase and 5-nucleotidase were determined in sera and starch gel by the method of Dixon and Purdom(1) with the following modifications: 0.3 ml of 0.05 M $MgSO_4$ was substituted for 0.3 ml of water in the pH 9.3 and 7.5 β-glycerophosphate substrate systems, while 0.3 ml of 0.4 M $MgSO_4$ was used for the pH 7.5 adenosine-5-phosphate substrate system. Serum proteins were separated by zone electrophoresis according to a modification of the method of Smithies(2,3). Idaho potato starch† was hydrolyzed for 70 minutes at 36.4°C and dried to a moisture content of 10.3%. The gel was prepared by heating 13.5 g of this soluble starch with 100 cc of 0.027 M borate buffer, pH 9.05. Electrophoresis was carried out at 12°C at 4.5 volts per cm for 16 hours. The gels were then scored in ½ cm sections and cut in half along the horizontal plane. One half was retained for protein staining while the second half was sectioned in ½ cm blocks and placed directly into tubes containing the respective buffer, substrate, and required Mg concentrations.

Results. In sera of both normal subjects and of patients with eleavted non-specific alkaline phosphatase, there appear to be 2 alkaline phosphatases. One of these migrates in the region of alpha-2-globulin and beta-lipoprotein, the other with the beta-globulin components of serum (Fig. 1). Approximately 60% of the alkaline phosphatase migrating with the alpha-2-globulin and beta-lipoprotein is made up of 5-nucleotidase. There is no 5-nucleotidase activity in the alkaline phosphatase that migrates with the beta-globulin fraction (Fig. 1). Total non-specific alkaline phosphatase and 5-nucleotidase recoveries in these starch gel blocks were 90% and 40% of the serum used, respectively. Since borate is a known inhibitor of non-specific alkaline phosphatase(4), we attempted to show the relationship of borate inhibition and its correction by addition of Mg ions. It can be seen from Table I that the inhibition of alka-

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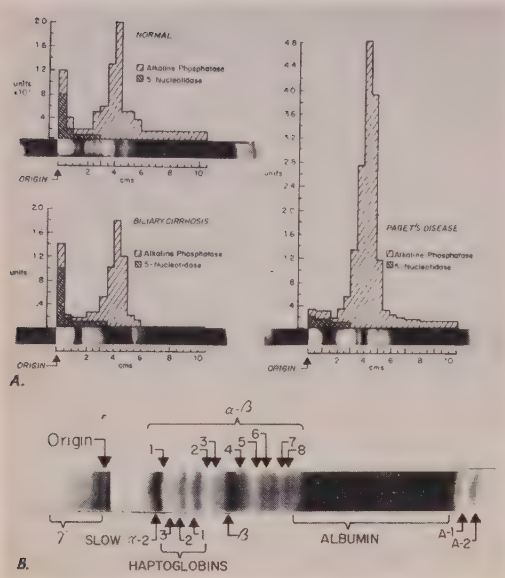

FIG. 1. Localization of 5-nucleotidase and non-specific alkaline phosphatase by starch gel electrophoresis. A. Demonstrating sites for these enzymes in normal individual and in patients with biliary cirrhosis and Paget's disease. B. Starch gel electrophoregram of normal serum for reference.

line-phosphatase is corrected by the Mg concentration used, while there is still 50% inhibition of the 5-nucleotidase even with 0.4 M Mg used in our test system. We feel that this accounts for the 40% yield of 5-nucleotidase found utilizing starch gel electrophoretic separations with 0.027 M borate buffer.

Discussion. Results of paper electrophoresis indicate that the locus of non-specific alkaline phosphatase coincides exactly with that of alpha-2-globulin(5,6). Recently, Fahey *et al.* have separated 2 alkaline phosphatases by an anion-exchange cellulose chromatographic method(7). Our results clearly demonstrate 2 electrophoretically separable alkaline phosphatases in both normal sera and sera of pathological states. One is found in the region of beta-lipoprotein and alpha-2-globulin, the other with beta-globulin. The alkaline phosphatase that coincided with the latter fraction of serum occurred in all cases studied and was increased strikingly in patients with Paget's disease and biliary cirrhosis. A similar but less exaggerated peak is seen in healthy children and in patients with Laennec's cirrhosis, obstructive jaundice due to stone and carcinoma, osteomalacia secondary to malabsorption, lymphoma, and sarcoidosis. Since the electrophoretic mobilities are consistently similar in all cases studied, and since there is no 5-nucleotidase present in this beta-globulin peak, it would appear that this enzyme represents alkaline phosphatase derived from bone. The remaining alkaline phosphatase migrates with the alpha-2-globulin and beta-lipoprotein and is made up of both 5-nucleotidase and non-specific alkaline phosphatase. Since there is no 5-nucleotidase in bone, and since level of serum 5-nucleotidase was normal in diseases of the bone, it would appear that the source of this enzyme is probably the liver.

Summary. 1. By means of starch gel electrophoresis, 2 alkaline phosphatases were demonstrated, one migrating in the region of alpha-globulin and β-lipoprotein and the other with beta-globulin. 2. 5-nucleotidase consti-

TABLE I. Effect of Magnesium Ions on Inhibition of Serum 5-Nucleotidase and Non-Specific Alkaline Phosphatase by Borate.

Enzymes		Conc. borate, M	Conc. Mg, M	Units of enzyme activity	% inhibition of control
Alk. p'tase,	pH 9.3	0	5×10^{-2}	4.3	0
5-Nucl.	7.5	0	4×10^{-1}	1.64	0
Alk. p'tase	9.3	$.625 \times 10^{-2}$	5×10^{-2}	4.3	0
5-Nucl.	7.5	"	4×10^{-1}	1.2	24
Alk. p'tase	9.3	1.25×10^{-2}	5×10^{-2}	4.3	0
5-Nucl.	7.5	"	4×10^{-1}	1.2	24
Alk. p'tase	9.3	2.5×10^{-2}	5×10^{-2}	4.2	2
5-Nucl.	7.5	"	4×10^{-1}	.8	50
Alk. p'tase	9.3	2.5×10^{-2}	0	3.4	21
5-Nucl.	7.5	"	0	.5	70

Alk. p'tase = Alkaline phosphatase. 5-Nucl. = 5-Nucleotidase.

tuted 60% of the former, but was not present in the latter. 3. Although alkaline phosphatase which migrated with beta-globulin was elevated in both diseases of bone and liver, it is thought to be derived from bone since it had no 5-nucleotidase. 4. Since 5-nucleotidase is not contained in bone and is not elevated in diseases of the bone, it is thought to be derived from the liver.

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Effect of Neomycin on Serum Cholesterol Level of Man.* (24569)

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During a clinical study of the effect of various dietary fats on the serum lipids of a group of patients with coronary atherosclerosis, it was observed that oral administration of neomycin to a patient with a gastrointestinal infection due to Salmonella Tennessee was associated with a significant fall in serum cholesterol concentration. The decrease in serum cholesterol level occurred at a time when the patient had clinically recovered from the infection although the organism was still present in the stools. To evaluate the significance of this observation, the effect of neomycin administered orally on the serum lipids was studied in 10 additional patients, none of whom were known to have disease of the gastro-intestinal system.

Method. The study group consisted of 6 male and 4 female patients ranging in age from 21 to 72 years. Nine were hospitalized and one was observed as an outpatient. Clinical diagnosis of each subject is listed in the Table. Total serum cholesterol levels were determined twice weekly in each patient by the method of Abell *et al.*(1). The ester fraction was determined by the method of Schoenheimer and Sperry(2); and total serum lipids by a gravimetric procedure(3). Five of the hospitalized individuals were on the regular hospital diet in which 45% of the calories are derived from fat. Of the remaining 5 patients, one (Nas) received a diabetic diet of C 180, P 80, F 80 g without insulin; one (Ber), with familial hypercholesterolemia, was on a low fat diet in which 25% of the calories were derived from fat; 2 patients were maintained on a similar low fat diet after a preliminary period of 6 weeks on the regular hospital diet; and the outpatient ate her usual diet without restriction during the study. Average pretreatment serum lipid values were established during periods of 5 weeks or longer prior to oral administration of neomycin. Neomycin was given as Mycifradin Sulfate®.† This preparation contains 70% neomycin sulfate. The doses of medication in this study represent the weight of Mycifradin Sulfate. Six patients were given 2 g each day. The remaining 4 patients received from 0.5 to 2 g daily. The period of neomycin administration varied from 3 to 16 weeks. If the amount of drug was varied, it was usually done so after 4 week periods at each dose level.

Results. The results of oral administration of neomycin on the serum cholesterol level are

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† The authors are indebted to Upjohn Co. for generous supply of Mycifradin Sulfate.

TABLE I. Effect of Neomycin on Average Serum Cholesterol Level (mg/100 cc), in 10 Patients.

Age	Sex	Diagnosis	Regular diet	Low fat diet	Neomycin									
					0.5 g	Wk*	% fall	1 g	Wk*	% fall	1.5 g	Wk*	% fall	2 g	Wk*	% fall
59	♀	Cerebral throm.	233		189	4	19	198	4	15	174	4	25			
58	♂	Hypertensive heart dis.	237		202	6	15	186	10	22						
69	♂	Cerebral throm.	206	194				172	4	11	157	6	19			
50	♂	Rheumatic heart dis., diabetes mel.	241		204	4	14							331	10	18
52	♀	Fam. hyperchol., coronary art. disease		405										189	4	19
72	♂	Coronary art. disease	262	233										209	4	21
52	♂	Periph. neuritis	266											279	6	24
21	♀	Fam. hyperchol.	366											367	3	17
36	♀	*Idem*	442											134	3	29
64	♂	Coronary art. disease	188													

* Weeks on neomycin.

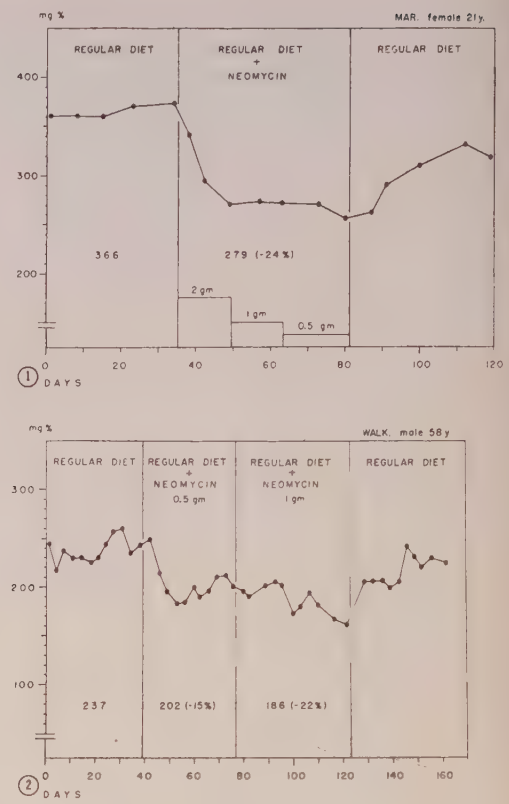

FIG. 1 and 2.

summarized in the Table. In all the patients, the average serum cholesterol level decreased following oral administration of neomycin. The decrease varied from 17 to 29% (average 22%), when 1.5 or 2 g of neomycin was given daily. At the dose level of 0.5 g of neomycin daily, average decrease in serum cholesterol varied from 14 to 19%; at the 1 g dose level average serum cholesterol diminished from 11 to 22%. Two to 3 weeks of oral neomycin administration were required before the level of serum cholesterol decreased to its low point. Increasing the amount of neomycin from 0.5 to 2 g daily resulted in a greater fall in serum cholesterol level. The lowering effect of neomycin could be maintained for the duration of the study, a period as long as 16 weeks, at the dose level of 1.5 to 2 g daily. Serum cholesterol levels returned to control levels within 2 weeks after the medication was discontinued.

Figs. 1 and 2 demonstrate the effect of oral neomycin in 2 patients. In Fig. 1 (Patient

Mar) serum cholesterol level averaged 366 mg % during the pre-treatment period and fell to average 279 mg % while on neomycin. The patient received 2 g of neomycin daily for the first 2 weeks, 1 g daily for the next 2 weeks, and finally 0.5 g daily for 2 weeks. The fall in the serum cholesterol was maintained although amount of medication was reduced. In Fig. 2 (Patient Walk) it can be seen that increasing the amount of neomycin from 0.5 g daily to 1 g daily resulted in a further decrease in serum cholesterol level.

The esterified fraction of serum cholesterol decreased in the serum in proportion to the total serum cholesterol in the 3 patients studied. Total serum lipids decreased in each patient, average fall for the group being 23% during the neomycin period. C/P ratio remained approximately unchanged during the study.

Two patients were subsequently given 60 mg of neomycin intramuscularly daily for 2 weeks without appreciable change in serum cholesterol level. This amount of neomycin was calculated to exceed that proportion of oral neomycin (3%) which is absorbed from the gastrointestinal tract(4).

No significant side effects occurred as the result of oral neomycin but mild transitory diarrhea occurred in 4 of the 10 patients at the 2 g dosage. Otherwise, the patients' clinical status remained unchanged during the period of study. The renal status as measured by urinalysis and blood urea nitrogen determinations and liver function as determined by cephalin flocculation and serum bilirubin tests were unaltered.

Comment. The mechanism of action of neomycin in lowering serum cholesterol concentration is unexplained. It is known that 97% of the orally administered drug is eliminated unchanged in the stool. It is possible that the effect of neomycin on the intestinal bacterial flora or upon certain enzyme systems of the gastro-intestinal tract may be responsible for the decrease in serum cholesterol level. Further studies utilizing antibiotics other than neomycin are in progress.

Summary. Oral administration of neomycin was associated with a significant decrease in serum cholesterol concentration of all of the 10 patients studied. On 1.5 to 2 g of neomycin daily, mean serum cholesterol level in each patient was decreased from 17 to 29%, average fall for the group was 22%. The fall in serum cholesterol level was maintained for the duration of drug administration which varied from 3 to 16 weeks. At a daily dose level of 0.5 to 1 g of neomycin similar but less marked falls in the serum cholesterol level resulted.

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Mobilization of Fatty Acids by Epinephrine in Normal and Hypophysectomized Rhesus Monkeys.*† (24570)

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The demonstration by Dole(1), Gordon and Cherkes(2) and others(3) that epinephrine markedly increases the concentration of circulating non-esterified fatty acids

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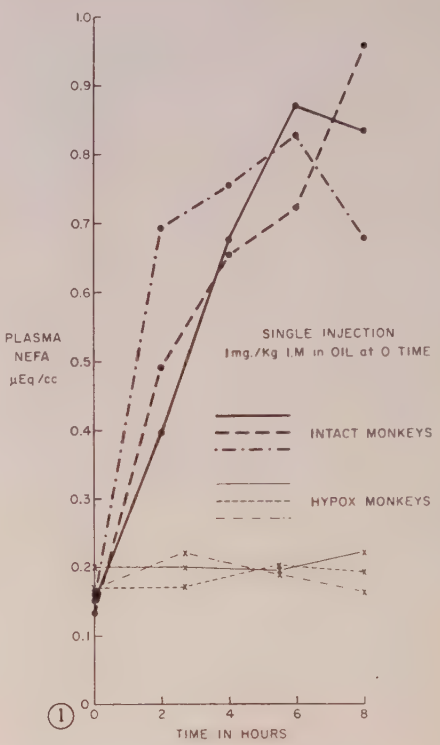
‡ Public Health Service Predoctoral Research Fellow, Nat. Cancer Inst.

(NEFA) was confirmed in *in vitro* systems (4,5) suggesting a direct effect of epinephrine on adipose tissue with a resultant release of NEFA to the perfusing medium. In the course of studies designed to investigate possible interrelations between this effect of epinephrine and the previously described action of fasting(1,2,6) and growth hormone administration(3), on plasma NEFA concentrations the unexpected observation was made that epinephrine, in contrast to growth hormone, was ineffective in the hypophysectomized rhesus monkey. The following report deals with the role of various endocrine factors in the NEFA mobilizing action of epinephrine.

Materials and methods. Normal and hypophysectomized rhesus monkeys (*Macaca mulatta*) of both sexes were utilized in this study. Hypophysectomy, using the parapharyngeal approach, was performed at least 2 months before initiation of experiments. All animals were maintained on a natural diet supplemented by a commercial monkey ration(7). A 10% glucose solution was available to hypophysectomized monkeys in addition to tap water except during experimental periods. Epinephrine (1 mg/kg I.M.) was administered in oil as a single injection. Control animals received an intramuscular injection of an equal volume of peanut oil. Epinephrine injections were made one hour following a standardized feeding, the animals being fasted throughout the remainder of the experiment. Plasma NEFA concentrations were determined by the method of Dole(1) and blood glucose by the method of Nelson(8) from samples drawn from the femoral vein into heparinized syringes.

Results. A single intramuscular injection of epinephrine (1 mg/kg in oil) produced a 5- to 6-fold increase in plasma NEFA concentration in the intact animals. In sharp contrast, the same dose of epinephrine, administered under identical experimental conditions, was without effect in hypophysectomized monkeys (Fig. 1). The hyperglycemic effect of epinephrine, on the other hand, was noted in both groups of animals.



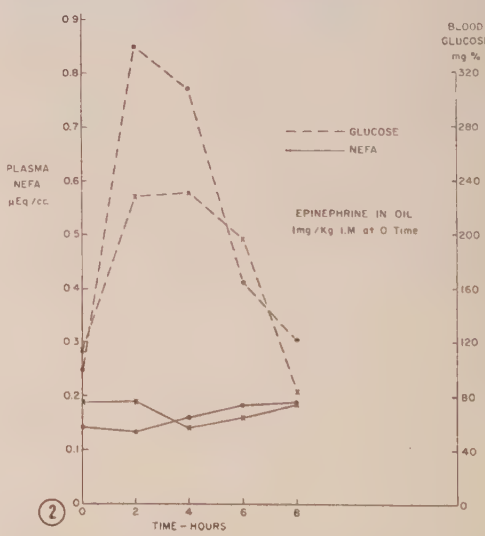


FIG. 1. Effect of epinephrine on plasma NEFA concentrations in intact and hypophysectomized rhesus monkeys.

FIG. 2. Effect of cortisol acetate pretreatment (3 mg/kg/day for 6 days) on NEFA response to epinephrine in hypophysectomized rhesus monkeys.

Comparable findings have been reported with reference to the hyperglycemic action of epinephrine. The hyperglycemic response to epinephrine is attenuated in hypophysectomized and adrenalectomized animals. Pretreatment of such animals with adrenal corti-

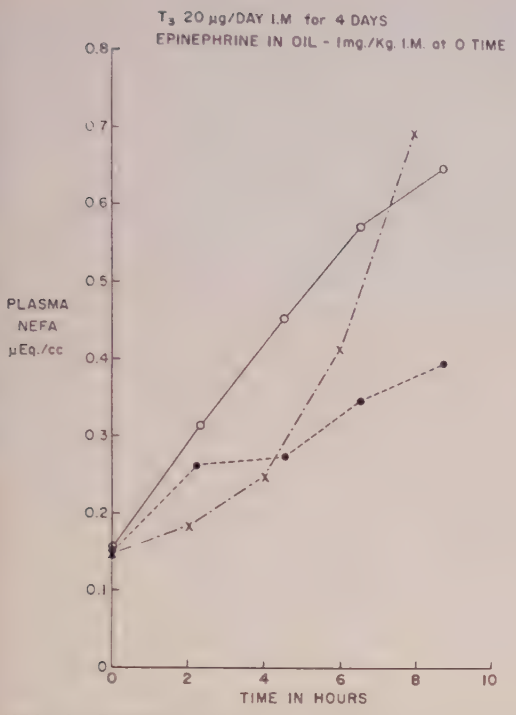


FIG. 3. Response of hypophysectomized monkeys to epinephrine following pretreatment with l-triiodothyronine.

cal steroids restores the hyperglycemia following epinephrine administration to normal (9). With these observations in mind, hypophysectomized monkeys were given daily injections of cortisol acetate (3 mg/kg I.M.) for 6 days and NEFA response to epinephrine determined on the seventh day. While the hyperglycemic effect of epinephrine was fully evident, no rise in plasma NEFA concentration was observed (Fig. 2).

Pretreatment of hypophysectomized monkeys with prolactin (200 I.U. per day for 4 days) and ACTH (40 units per day for 4 days in gelatin) similarly failed to restore NEFA response to epinephrine. Pretreatment with monkey growth hormone (1 mg/kg per day for 4 days), however, caused a slight rise in plasma NEFA concentrations when epinephrine was administered.

The known TSH contamination of the monkey growth hormone and the hypothyroid state of the hypophysectomized monkeys suggested pretreatment with TSH and thyroid hormone. Pretreatment with 5 units of TSH per day for 4 days preceeding the epinephrine injection partially restored the NEFA response. Pretreatment with 20 μg-l-triiodothyronine per day for 4 days restored ability of hypophysectomized monkeys to respond to epinephrine in terms of an increase in plasma NEFA concentrations (Fig. 3). This was sustained for several weeks following cessation of thyroid therapy.

These observations suggest that the inability of hypophysectomized rhesus monkeys to respond to epinephrine by a mobilization of NEFA can best be explained in terms of the hypothyroid state of these animals and that optimal thyroid function is requisite for the NEFA mobilizing action of epinephrine. As mentioned above, such does not appear to be the case for growth hormone and fasting since both hypophysectomized and intact animals respond to these experimental situations.

Summary. Mobilization of NEFA by epinephrine in rhesus monkeys is abolished by hypophysectomy while the hyperglycemic response is retained. Pretreatment of hypophysectomized monkeys with cortisol acetate, ACTH, prolactin and growth hormone fails to restore response to epinephrine in terms of NEFA mobilization. Pretreatment with TSH or triiodothyronine, however, produced a restoration of the response. It is concluded that optimal thyroid function is requisite for the NEFA mobilizing action of epinephrine *in vivo*.

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Effect of Olive Oil and Squalene on Cholesterol Mobilization in the Rat.* (24571)

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Squalene is considered a precursor in biosynthesis of cholesterol(1,2). Olive oil is unique in that it is the only commonly used vegetable fat which contains squalene in appreciable amounts (31% to 64% of the nonsaponifiable fraction, or about 0.5% of the oil)(3,4,5). It also contains a higher percentage of oleate and a lower percentage of linoleate than do corn, soy and cottonseed oils. Coconut oil, in contrast, contains little oleate or linoleate, but it contains a high proportion of glycerides of saturated acids with shorter chain length than palmitic acid. Young female rats fed 10% olive oil with 1% cholesterol had higher serum cholesterols than did matched groups of rats fed other oils cited above, with the same synthetic diets. High serum cholesterol figures were observed only in cholesterol-fed females and not in cholesterol-fed males. It seemed possible, therefore, that when cholesterol synthesis was inhibited by cholesterol feeding, dietary squalene might be serving either as a stimulant of, or a precursor for, synthesis of some ovarian hormone.

Materials and methods. Intact rats. Squalene,† at approximately maximum level furnished by olive oil, was added to diets containing 10% cottonseed and 10% coconut oils, respectively.‡ Diets containing 10% of new and specially prepared olive oil were also fed, with and without cholesterol. Weanling rats in groups of 10 males and 10 females were given the diets, (Table I) for 7 weeks. They were then sacrificed, and liver and serum lipids were determined. Methods were those reported previously(7).

Results. Data for serum and liver lipids and cholesterol are given in Table I. Serum cholesterols were again much higher ($P < 0.01$) in olive oil-cholesterol-fed females than in companion groups fed either cottonseed or coconut oils. Serum levels of cholesterol in females fed squalene and cholesterol with cottonseed oil showed some increase over the group fed cholesterol only. When squalene was given with coconut oil and cholesterol, increases approached significance ($P < 0.05$). Serum values were raised only in cholesterol-fed females.

Both liver lipid and liver cholesterol values (mg/liver) were significantly higher ($P < 0.01$) in squalene- and cholesterol-fed male as well as female rats given coconut oil than in their companion groups with cholesterol but without squalene. Rats of both sexes given olive oil with cholesterol likewise had significantly higher liver lipids and cholesterol than did those given cottonseed or coconut oil with otherwise identical diets. The higher values for liver lipids and cholesterol observed when squalene was given to cholesterol-fed males suggested the possibility that squalene might in some way be accelerating retention of liver glyceride and cholesterol. Whether this was a direct effect or one mediated by a hormone was not clear.

Castrates. To test whether squalene affected the level of serum and liver cholesterol in the absence of hormones secreted by the gonads, male and female castrates were studied. Rats of each sex were placed, at

* Supported in part by grant, Nat. Heart Inst., U.S.P.H.S.

† Eastman—Technical grade.

‡ The diets contained: 10% albumin; 5% vit-free casein; 10% fat; .05% squalene (when fed); 1% vit. B mix; 1% fat-soluble vit. mix (in cottonseed oil); 1% cholesterol (when fed); and sucrose to 100%. The vitamins furnished/kg of diet were: 15,000 USP units A; 1,000 USP units D; 5 mg menadione; 400 mg mixed tocopherol; 4 mg thiamine; 4 mg riboflavin; 2 mg pyridoxine; 10 mg calcium pantothenate; 2 mg folacin; 1.5 mg biotin; 10 mg p-aminobenzoic acid; 10 mg niacin; 100 mg ascorbic acid; 500 mg inositol; and 900 mg choline. This diet differed from the 10% fat diets described in(3) in that it furnished a slightly higher percentage of choline.

TABLE I. Lipids and Cholesterol: Intact Rats. Means and stand. errors. (10 rats/group.)

Fat in diet*	Sex	Gain body wt, g	Liver Wt, g	Liver Total cholesterol % moist wt	Liver Total cholesterol mg/liver	Liver Free cholesterol, % moist wt	Liver Lipids % moist wt	Liver Lipids mg/liver	Serum Total cholesterol, mg %
Ol	♂	223	9.6	.39 ±.03†	38 ± 4.3	.23	5.2 ± .19	500 ± 49	88 ± 6
	♀	155	5.9	.30 ±.01	18 ± 1.1	.25	5.1 ± .18	300 ± 17	72 ± 5
Ol + C	♂	240	11.9	3.97 ±.29	474 ±54.0	.33	15.1 ±1.7	1814 ±262	96 ± 8
	♀	162	6.7	4.23 ±.49	290 ±41.0	.36	13.9 ±1.4	946 ±116	272 ±47
CsO + C	♂	209	9.6	2.80 ±.30	270 ±34.0	.31	12.5 ± .7	1203 ± 90	77 ± 3
	♀	157	6.5	2.05 ±.33	134 ±25.0	.33	9.7 ± .9	624 ± 72	114 ±15
CsO + C + Sq	♀	167	6.9	3.10 ±.30	214 ±21.0	.35	12.8 ±1.0	890 ± 76	147 ±19
CnO	♂	222	8.5	.30 ±.01	25 ± 1.4	.23	4.7 ± .3	398 ± 29	71 ± 3
	♀	155	5.4	.25 ±.01	14 ± 3.7	.24	5.0 ±3.7	260 ± 22	77 ± 6
CnO + Sq	♂	223	10.2	.37 ±.04	36 ± 4.8	.22	5.6 ± .3	546 ± 48	76 ± 5
	♀	138	6.0	.31 ±.04	19 ± 2.4	.27	6.0 ± .8	368 ± 56	76 ± 5
CnO + C	♂	232	9.5	1.47 ±.02	135 ± 2.0	.29	8.0 ± .7	735 ± 65	96 ± 8
	♀	157	6.1	.79 ±.01	48 ± 5.0	.32	6.1 ± .6	377 ± 49	117 ±11
CnO + C + Sq	♂	224	11.5	2.53 ±.35	303 ±52.0	.30	16.9 ±2.8	2092 ±452	82 ± 8
	♀	156	6.5	1.93 ±.21	125 ±13.0	.33	8.8 ± .6	569 ± 38	153 ±13

* Abbreviations: Ol = olive oil; C = cholesterol; CsO = cottonseed oil; Sq = squalene; CnO = coconut oil.

† Stand. errors: $\frac{\sqrt{\frac{\Sigma d^2}{n-1}}}{\sqrt{n}}$.

weaning, on the 10% coconut oil diets (a) without, and (b) with added cholesterol. Subgroups, on each diet, were fed squalene. After approximately 3 weeks, the animals were gonadectomized. They were continued on respective diets for 4 more weeks, so that age and total time on diet were comparable with those of intact rats.

The data in Table II show that addition of squalene to a cholesterol-rich diet had little effect on serum or liver cholesterol of castrates of either sex. The data for intact and for castrate rats are not strictly comparable because of consistently higher liver lipid and cholesterol values of cholesterol-fed castrates. Nevertheless, it is significant that intact animals fed squalene with cholesterol had liver cholesterol values twice as high as those of intact animals fed cholesterol only, while, in castrates, there were no significant differences between liver cholesterols of squalene-cholesterol-fed groups and those fed cholesterol only. Serum values for castrates were not significantly affected by addition of squalene to cholesterol-rich diets. Female castrates did, however, have higher mean serum cholesterols than male castrates. Variations within groups were considerable.

Discussion. The structure of squalene offers many possibilities for formation of cis and trans isomers. It is possible that the commercial squalene which was fed may have had the lesser effect on serum cholesterol because of presence of unnatural isomers. The squalene of olive oil might be different and possibly more active.

On the other hand, data for olive oil-fed rats can hardly be interpreted without consideration of the high oleate content of this oil (75%-85%). Cottonseed oil is richer in linoleate (about 50%), but coconut oil owes its low melting point to its content of saturated acids of short to medium chain length. Dietary oleate and linoleate both seem to facilitate deposition of liver cholesterol in the rat, and large percentages of oleic acid have been found in both liver cholesterol esters and glycerides. The observed effects of olive oil might have been a summation of a specific action of squalene, plus a mass effect of a large supply

TABLE II. Lipids and Cholesterol: Castrates. 10% coconut oil diet. Means and stand. errors.

Diet supplement	No. & sex	Wt gain, g	Liver wt, g	Liver: Total cholesterol % moist wt	Liver: Total cholesterol mg/liver	Liver: Total lipids % moist wt	Liver: Total lipids mg/liver	Liver: Free cholesterol, % moist wt	Serum cholesterol, mg %
None	7 ♂	206	8.2	.27 ±.01*	22 ± 1.2	4.3 ±.3	355 ± 28	.23 ±.01	79 ± 7
	8 ♀	176	6.7	.27 ±.01	18 ± 1.0	5.7 ±.4	378 ± 24	.23 ±.01	80 ± 4
Squalene only	14 ♂	201	7.5	.29 ±.01	22 ± .9	4.9 ±.1	369 ± 12	.24 ±.01	68 ± 3
	12 ♀	182	6.8	.30 ±.01	20 ± .8	5.1 ±.2	345 ± 13	.24 ±.01	86 ± 5
Cholesterol only	8 ♂	199	8.9	2.50 ±.30	226 ±38.0	10.1 ±.6	904 ± 79	.31 ±.02	102 ±15
	8 ♀	180	7.6	2.00 ±.20	154 ±17.0	9.2 ±.4	700 ± 41	.30 ±.01	129 ±19
Cholesterol + squalene	11 ♂	203	9.1	2.23 ±.23	200 ±20.0	9.1 ±.6	811 ± 47	.30 ±.01	94 ± 9
	13 ♀	170	7.2	1.71 ±.10	122 ±11.0	8.4 ±.5	599 ± 39	.26 ±.02	134 ±16

* Stand. errors: $\frac{\sqrt{\frac{\Sigma d^2}{n-1}}}{\sqrt{n}}$.

of oleate on absorption and esterification of cholesterol and accumulation of glyceride.

Coconut oil furnishes very little oleic acid. Moreover, a considerable proportion of its fatty acids of short chain length can be expected to be absorbed *via* the portal circulation, rather than to accompany cholesterol through the lymphatics. That feeding squalene increases liver cholesterol in the coconut oil-fed animals is, therefore, the more perplexing, as is its effect on serum cholesterol of females only. The data for coconut oil-cholesterol-fed females seem reasonable only if we postulate that, where cholesterol synthesis is inhibited by cholesterol feeding, squalene is a more direct intermediate than cholesterol for synthesis of an ovarian hormone. This hormone, like estradiol(6), might raise the level of circulating cholesterol. It would also be necessary to assume that squalene either directly influenced liver lipid deposition, or that another hormone-assisted mechanism was active in producing high liver lipid and cholesterol values in male rats fed squalene with coconut oil and cholesterol. The possibility cannot, of course, be ruled out that some very active impurity may have been present both in the olive oil and in the squalene fed.

The large standard errors of serum cholesterol values for olive oil- and squalene-fed females were due to the fact that three-fourths of the animals had higher than the mean values given, while the rest were within normal range for the diet without squalene. The above might be expected if the effect of squalene on serum cholesterol were felt only during certain phases of the estrous cycle.

Summary. Much higher values for serum cholesterol were observed in 2 series of female rats fed 1% cholesterol with diets containing 10% olive oil than in the series fed like diets made up with a number of other vegetable fats. Olive oil-fed males did not have high serum cholesterols, but did have high liver lipids and cholesterol. An attempt to duplicate the effect of olive oil by adding equivalent amounts of commercial squalene to diets containing 10% cottonseed oil and 10% coconut oil is reported. The squalene-fed females on diets with added cholesterol had higher serum cholesterols than did those fed cholesterol only. Values were not nearly so high as in the rats fed olive oil. Both males and females fed squalene with cholesterol and coconut oil had significantly higher liver lipids and cholesterol than did littermates fed only coconut oil and cholesterol in the same amounts. Little or no effect attributable to squalene was observed in castrates. The possible effect of squalene as a stimulant of secretion of gonadal hormones is discussed.

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Phosphate Metabolism in Nutritional Muscular Dystrophy and Hyperthyroidism.* (24572)

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Impairment of phosphorylation of creatine has been suggested as the cause of creatinuria of both hyperthyroidism and Vit. E deficiency (1,2). In hyperthyroidism it is possible to demonstrate *in vitro* the uncoupling of oxidative phosphorylation in mitochondria(3), but recent evidence indicates that this may be due to a primary effect on the mitochondrial membrane(4). A method for measuring oxidative phosphorylation *in vivo* is not available, but it is possible to determine the entry of radioactive phosphorus into tissue phosphates. This approach is used in the following experiments to measure phosphorylation of creatine.

Methods. Vit. E deficiency was produced in white male New Zealand rabbits, weighing approximately 500 g, and in weanling Sprague-Dawley rats of both sexes by feeding previously described purified diets deficient only in Vit. E(5,6). Oral supplementation of control animals with Vit. E and general handling of animals were also the same as in previous studies(5,6). Rabbits on the deficient diet exhibited signs of muscular dystrophy after 3 to 4 weeks, then were used in experiments. The group of rats given Vit. E-deficient diet exhibited marked creatinuria and significant decrease in growth rate when compared to normal rats, and there was microscopic evidence of muscular dystrophy after 7 months of feeding, then chosen for the experiments. Two other groups of weanling Sprague-Dawley rats of both sexes were placed on regular laboratory chow diet. One group served as controls while hyperthyroidism was induced in the other group by daily subcutaneous injections of 0.5 mg of sodium thyroxinate for 21 days. The thyroxine-injected rats exhibited usual signs of hyperthyroidism when used in experiments. All animals were given 0.4 mc of $P^{32}O_4$ in dilute HCl/kilo body weight. Specific activity of the $P^{32}O_4$ was approximately 100,000 mc/g. Control and Vit. E-deficient rabbits were given P^{32} intravenously and killed 30 minutes later by large intravenous doses of pentobarbital. Immediately after death the animals were transferred to the cold room where samples of blood and skeletal muscle were obtained for analysis. The rats were handled in the same manner except that injections were given intraperitoneally. Control and Vit. E-deficient rats were killed 30 minutes after injection of P^{32} and control and hyperthyroid rats were killed one hour following injection of P^{32}. The scheme of fractional hydrolysis and precipitation of tissue phosphates devised by Sacks(7) was used to isolate serum inorganic phosphate, skeletal muscle inorganic phosphate, and the phosphate of creatine phosphate and adenosinetriphosphate (ATP). Portions of each specimen were used for phosphorus determination by the method of Fiske and SubbaRow and for counting with an end window Geiger counter.

Results. The skeletal muscle inorganic phosphate concentrations were in good agreement with the values given by Threlfall(8) and no differences were observed between the

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TABLE I. Mean Specific Activities (c.p.m./μmole P) of Plasma and Skeletal Muscle Phosphates.

Exp. group	No. of animals	Time after inj., min.	Plasma inorg. PO_4	Skeletal muscle inorg. PO_4	Creatine PO_4	ATP PO_4
Control rabbits	5*	30	49,410	1,559	586	689
Vit. E-def. rabbits	5*	30	50,057	5,023	1,425	2,265
P value†				<.01	<.05	<.01
Control rats (chow diet)	7	60	39,024	2,511	770	860
Hyperthyroid rats	5	60	27,215	2,828	1,312	1,510
P value				>.05	>.05	<.01
Control rats (purified diet)	4	30	85,225	2,528		
Vit. E-def. rats	4	30	93,033	5,495		
P value				<.01		

* Only 3 animals were used for isolation of plasma inorganic phosphate in these rabbits.
† Probability that differences observed were due to chance as determined by the t test.

various experimental groups.

Table I gives specific activities (S.A.) of the various phosphates. Plasma inorganic phosphate S. A. was altered appreciably only by hyperthyroidism and in each instance S. A. was considerably greater than that of tissue phosphates. This indicates that an equilibrium with tissue phosphates had not been reached.

Skeletal muscle inorganic phosphate S. A. was significantly increased in both Vit. E-deficient rabbits and rats, and unchanged in the hyperthyroid rats. S. A. of phosphate of ATP and creatine phosphate was significantly increased in hyperthyroid rats as well as in Vit. E-deficient rabbits.

Discussion. Perhaps the most important finding is the high skeletal muscle inorganic phosphate S. A. in Vit. E-deficient rabbits and rats, with no change in serum inorganic phosphate specific activities. The difficulty with interpreting this observation has been generally recognized in similar studies involving isolation of tissue phosphates(9,10). It should be noted that the usual correction for skeletal muscle extracellular space has not been made in recording our data. The validity of such a correction is doubtful, but if applied, it would accentuate the differences between control and Vit. E-deficient animals. Assuming that rate of transfer of inorganic phosphate from blood to the extracellular fluid is not affected by Vit. E deficiency and that size of extracellular space is unchanged, the finding of a high skeletal muscle inorganic phosphate S. A. indicates a high rate of turnover between extracellular and intracellular phosphate since the rate of esterification of phosphate was not reduced.

The high S. A. of the phosphates of creatine phosphate and ATP phosphate in Vit. E-deficient rabbits agrees with the report of Ferdman(11), but probably only reflects a high intracellular inorganic phosphate S. A. If the skeletal muscle inorganic phosphate S. A. is accepted without correction for the extracellular inorganic phosphate, there is no decrease in turnover of creatine phosphate or ATP. On the other hand if a correction were made for extracellular phosphate it would be possible to conclude that there is a decreased turnover of creatine phosphate and ATP. It is therefore necessary to state that rate of phosphorylation of creatine and ATP in skeletal muscle is unchanged or decreased by Vit. E deficiency in the rabbit.

The phosphates of creatine phosphate and ATP also have a high S. A. in hyperthyroid rats. In this case interpretation of the data is less difficult because, even after correction for the extracellular phosphate, the turnover of these phosphates still appears increased. From this, it is concluded that there is no impairment of the phosphorylation of creatine and ATP in skeletal muscles of hyperthyroid rats.

Summary. 1) Rabbits and rats were made Vit. E-deficient by feeding a purified diet. Control animals received the same diet supplemented with Vit. E. Hyperthyroidism was

induced in rats by giving subcutaneous injections of sodium thyroxinate. Control and experimental animals from each group were injected with P^{32} in dilute HCl and specific activities of plasma inorganic phosphate, and skeletal muscle inorganic phosphate, creatine phosphate, and ATP phosphate determined. The S. A. of the plasma inorganic phosphate was unchanged by Vit. E deficiency and was decreased by hyperthyroidism. Skeletal muscle inorganic phosphate S. A. was increased by Vit. E deficiency and the difficulties in interpreting these data were discussed. The S. A. of phosphates of creatine phosphate and ATP phosphate were high in both Vit. E deficient rabbits and hyperthyroid rats. The significance of these findings was discussed in relation to turnover of creatine phosphate and ATP. 2) These experiments indicate that there is an elevated turnover of skeletal muscle intracellular inorganic phosphate and a normal or decreased turnover of skeletal muscle creatine phosphate and ATP in Vit. E deficiency. In contrast there appears to be no depression in turnover of creatine phosphate and ATP in hyperthyroid rats.

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Experimental Colloid Goiter in the Hamster.* (24573)

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Fifty years have passed since David Marine demonstrated the relationship of iodine to goiter and expressed the belief "that goiter must be classed with nutritional disturbances (diseases) and that as such it will take its place in nosology along with chlorosis, rachitis, osteomalacia, etc.,"(1). This statement was based on morphologic and chemical studies of various types of goiter occurring naturally in man and other species, together with effects produced by administration of iodine to dogs with hyperplastic or colloid types of goiter(2,3). Thus, in a very short time Marine and his collaborators were able to lay the foundations for rational treatment and prophylaxis of endemic goiter, which he and Kimball had conclusively demonstrated by 1920(4). In his experimental studies Marine depended on dogs in which endemic goiters had already developed. He formulated the pathogenesis of goiter as follows: iodine deficiency acting on a normal gland leads to hyperplasia, which continues unless iodine is administered; whereupon colloid accumulates in the thyroid, a state which represents "the nearest approach to normal glands that active hyperplasias can become"(2). Colloid goiter, with varying degrees of nodular hyperplasia and scarring, is the form which the endemic disease takes in man. This morphologic picture has, to our knowledge, never been produced in laboratory animals whose thyroid glands were normal initially. To be sure, thyroid enlargement has been observed: 1) in puppies born to females whose thyroids had been extirpated before

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pregnancy(5), 2) following ingestion of cabbage in rabbits(6), 3) after feeding iodine-deficient diets to rats(7), 4) as a result of administration of thiourea and other compounds to several species(8) and 5) following TSH administration(9). All such goiters are characterized by varying degrees of hyperplasia without the diffuse colloid change which is found in naturally-occurring disease. Colloid cysts have been described in rats treated with thiouracil for prolonged periods(10). The pathogenesis of colloid goiter as formulated by Marine has needed laboratory confirmation for some time. Undoubtedly this gap in our knowledge has contributed to the confusion which has surrounded the subject of endemic goiter in the human for so many years. One of our programs has been to attempt to reproduce certain human deficiency disease syndromes in experimental animals (rats, rabbits, guinea pigs, hamsters and monkeys). During our studies, utilizing diets composed predominately of corn, goiter has been observed(11). Among species thus far studied the hamster appears to be the most suitable experimental subject. This communication records the successful production of colloid goiter in this animal.

Materials and methods. Hamsters weighing 75 to 140 g at beginning of experiment have been placed on 2 diets: (1) ground whole unenriched corn and (2) a modified Remington type mixture(7) consisting of ground corn, 76; wheat gluten, 20; sodium chloride, 2 and calcium carbonate 2; 500 mg of a complete vitamin mixture were added to each kilo of diet No. 2. These diets contain 1.0 and 2.5 μg iodine/100 g, respectively.* For controls, potassium iodide was added to each diet in concentration of 10 mg/kilo. This concentration in the diet was used for repletion studies as well. Food and tap water were allowed *ad lib.* Animals were kept in screen bottom cages in a well-ventilated room at constant temperature (78°F). Male and female animals were maintained on these diets for varying periods to 350 days. The thyroid glands of 54 deficient and 12 control animals have now been studied. Thyroid glands and other tissues were fixed in neutral 4% formalin, dehydrated in alcohols, imbedded in paraffin, cut and stained with hematoxylin-eosin or the periodic acid-Schiff technic.

Results. Response of thyroid gland to the 2 diets is similar save that alterations appear sooner and are more extensive on diet No. 2, since growth of animals is more satisfactory. The normal thyroid gland of the hamster weighs about 7 mg and is composed of follicles having an average diameter of approximately 45 μ. The cells lining the follicles are low cuboidal; eosinophilic colloid is abundant. Blood vessels are not prominent. Accumulations of fatty tissue are found within the gland (Fig. 2).

After 1 to 2 weeks on the deficient diet no thyroid enlargement is found, though grossly both lobes are more prominent because of increased vascularity. By the end of 2nd week, microscopic examination reveals that colloid has disappeared from the follicles, which are lined by cuboidal epithelium; dilated blood vessels separate the follicles. As time goes on, such vascularity becomes even more prominent; sometimes follicles appear to be virtually floating in a pool of blood. So, too, the epithelial cells increase in number and become more columnar, projecting into the lumens of the colloid-depleted follicles. By 125 days the enlarged glands consist of hyperplastic follicles surrounded by prominent vascular channels. The epithelium projects into the follicle as papillary tufts. In places, formation of intrafollicular follicles (12) is seen. At this stage a few follicles may contain eosinophilic colloid. From then on to 325 days, hyperplasia becomes extensive with more infolding of the epithelium. In addition more follicles contain colloid; these are lined by less columnar epithelium. In none of the glands do more than about 10% of the follicles contain colloid.

The reappearance of colloid in follicles with time led us to attempt to accelerate this reaction by administration of iodine. When animals which have been on diets for 125 days are given iodine, the follicles fill up with colloid; the epithelium naturally becomes cuboidal or flattened and the gland grossly and

* Analysis performed by Dr. J. V. Princiotto, Chemo-medico Consultants, Arlington, Va.

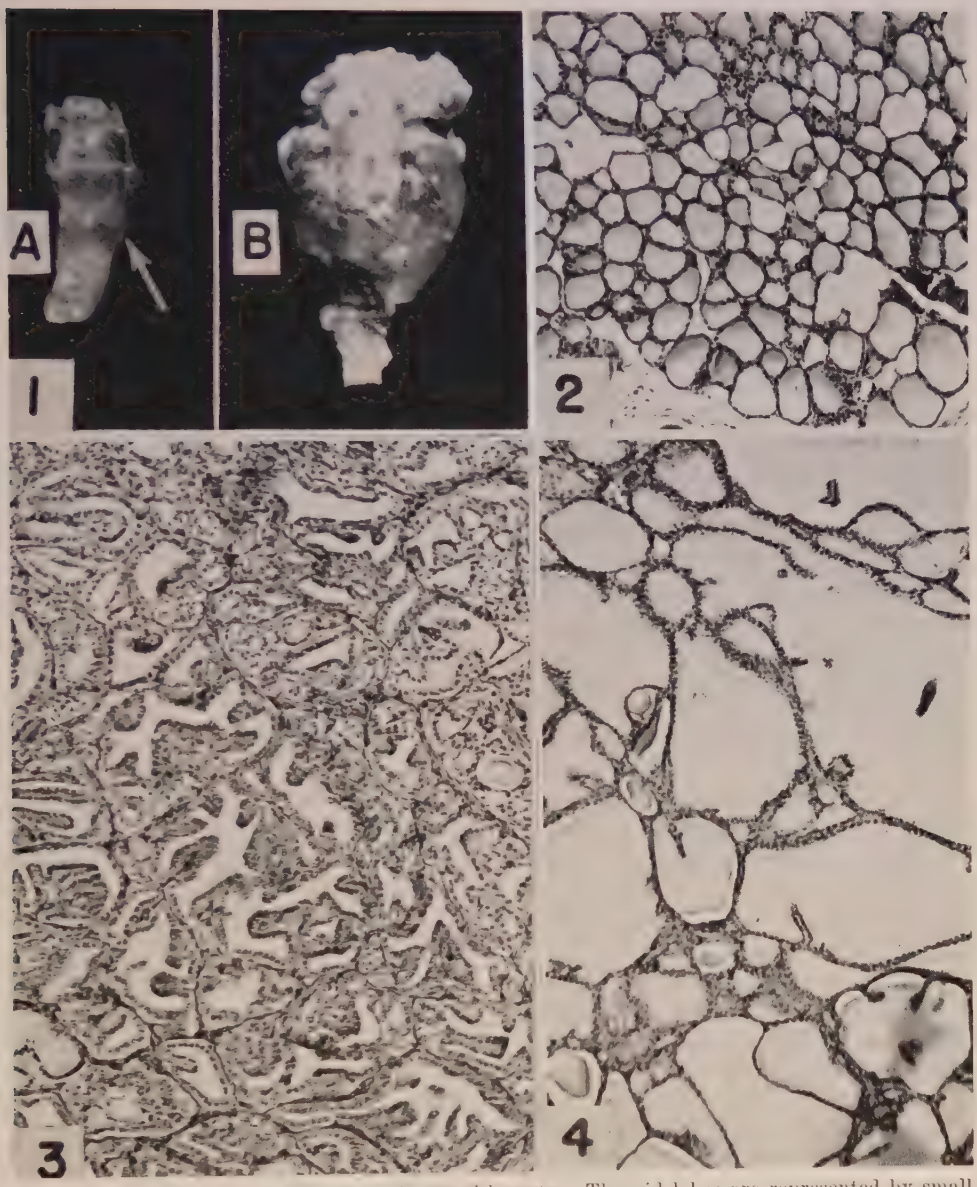

FIG. 1. A. Larynx and trachea of normal hamster. Thyroid lobes are represented by small structures (arrow) beneath laryngeal muscles. B. Colloid goiter developing after 325 days on iodine-deficient diet followed by 2 wk of iodine administration.

FIG. 2. Microscopic section of normal thyroid gland (×100).

FIG. 3. Hyperplastic gland after 325 days on iodine-deficient diet (×100).

FIG. 4. Colloid goiter developing in gland after 2 wk of iodine administration to gland similar to that seen in Fig. 3 (×100).

microscopically is less vascular. After 2 weeks of treatment remnants of papillary projections, some still lined by cuboidal epithelium, are found, but mainly the gland is composed of follicles, some as large as 150 μ in diameter, *e.g.*, 3 times normal. Small normal-sized follicles lined by flattened epithelium are found between these. If animals which have been on the deficient diet for over 300 days (Fig. 3) are repleted in similar fashion even larger colloid lined follicles are found (Fig. 4), some of which are 400 μ in diameter. Such

follicles still exhibit sprig-like projections into their lumens.

As might be expected, pituitary glands of the animals having hyperplastic glands are enlarged and contain thyrotrophic basophilic cells.

Discussion. Marine's fundamental contributions to our understanding of the pathogenesis of colloid goiter have all too briefly been mentioned above(1,2,3). The experimental observations described herein would seem to place Marine's conclusions, which were based on studies of naturally occurring goiters in man and animals, on a firm basis, since changes in thyroid structure from the normal to the hyperplastic gland and thence to the colloid form of goiter following administration of iodine can be followed, in the hamster at least, with ease. Now that colloid goiter can be produced in a small laboratory animal, a number of questions may be asked and in time perhaps can be answered. First and foremost is how permanent is the change since it is well-known that a colloid goiter in man does not ordinarily regress appreciably? Moreover, may the experimental colloid filled gland be made to regress under appropriate treatment? Is the colloid of the colloid goiter different chemically or physically from the normal? Is there a "point of no return" at some stage during the development of the change, *i.e.*, in early stages, at which the gland can no longer revert to its normal structure following iodine administration? May alternating cycles of deficiency and repletion lead to nodular changes such as are seen in human material? These and other questions are currently under investigation in this laboratory. Much of the confusion associated with our understanding of the pathogenesis of endemic goiter has undoubtedly been due to a failure to produce and study the disease in the laboratory(13). It is rather astounding that such has been the case, particularly when it is estimated(14) that the total number of cases of goiter in the world today "is probably not far short of 200 million"! It is hoped that these studies in the hamster may stimulate investigations in this and other species so that many of the unsolved problems can be elucidated.

Summary. Extreme hyperplasia of the thyroid gland has been produced in hamsters by placing them on iodine-deficient diets. When iodine is administered large amounts of colloid accumulate in the thyroid follicles, giving rise to the morphological picture of diffuse colloid goiter. Marine's concept of the pathogenesis of colloid goiter: normal gland ⟶ hyperplasia ⟶ (iodine treatment) ⟶ colloid goiter, appears to have been proved under laboratory conditions, in the hamster at least.

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Deficiency Diets in Young Growing Rats.* (24574)

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Choline biosynthesis is accomplished *in vivo* in the presence of serine, methionine, glycine, Vit B_{12} and folic acid. With ample serine and 1200 mg of methionine/100 g of ration, rats on choline deficient diet grow at normal rate and have normal liver lipids(1). However, Griffith states that hemorrhagic degeneration of kidney is wholly prevented by supplements of choline too small to affect deposition of liver lipids(2). An improved but reasonable working hypothesis involves the concept that the kidney requires a continuing source of choline for synthesis of lecithin(3). Hartroft *et al.* have shown pathological changes occur in major arteries of rats maintained on diets low in choline up to 126 days (4). These investigators indicate that choline is the essential dietary factor for cardiac muscle. Others believe methionine is the critical dietary factor for prevention of cardiovascular tissue damage in the rat(5). Force feeding of choline-containing diets devoid of methionine for 3 to 6 days induces fatty liver in adult female rats, but not in male rats(6). In the present study the effect of deficiencies of choline, methionine and cystine singly or paired was determined in young growing male rats. Organs were systematically examined for evidence of changes related to these deficiencies(7,8).

Materials and methods. One hundred and twenty young male rats of hardy, closely inbred modified Long-Evans strain, weighing 85 to 135 g, were employed. Animals were caged individually and kept at 70° to 80°F. The experimental dietary groups, each containing 15 animals, were established as follows: choline-deficient, methionine-deficient, methionine-cystine-deficient and cystine-deficient diets with 60 pair-fed controls (4 groups of 15 animals for each dietary regimen). Control diet consisted of 18% oxidized casein, 2% vitamin-free casein, 10% alpha soy protein, 35% corn oil, 4% salt mixture W (Nutritional Biochemicals Corp.), vitamin mixture(9) with addition/100 g of .85 g of choline and amino acid mixture containing 225 mg of tryptophan, 200 mg of tyrosine, 400 mg of cystine and 1200 mg of methionine. Sucrose was used to bring total diet to 100%. Experimental diets were obtained by eliminating from the control diet materials designated in the deficient diets. All animals were sacrificed at end of 6 weeks. Frozen sections (15 μ thick) of portions of thoracic aorta, kidney and liver of each animal were fixed in 10% formalin and examined for neutral fats employing oil red O stain and counterstained with Mayer's hemalum (Mallory, '52). In addition, portions of previously mentioned tissues and thymus, spleen, adrenals, pituitary and testes of each animal were fixed in Helly's fluid, sectioned in paraffin serially 7 μ thick, and stained with hematoxylin and eosin.

Results. Effect of dietary factors upon various organ weights are shown in Table I. Decreases in size of testes in methionine-deficient and methionine-cystine deficient groups are evident when compared to control pair-fed rats. Decrease of testes weight of cystine-deficient animals is statistically insignificant. Associated with these alterations are increases in thymic weights. The various diets resulted in no changes in splenic, pituitary and adrenal weights.

Grossly smaller testes were found in methionine-deficient and methionine-cystine-deficient animals. Microscopic examination of seminiferous tubules (Fig. 1) fails to show a compact and orderly arrangement. The seminiferous epithelium is similar in appearance to that of immature animals. Lack of sperm in the tubules was also noted.

Frozen sections of similar portions of thoracic aorta for all groups indicate that the amount of stainable fat does not differ significantly from that seen in pair-fed controls (Fig. 2).

* Supported by Grant from Lipotropic Research Fn.

TABLE I. Effects of Dietary Factors upon Various Organ Weights* of Intact Rats. (Means and stand. errors.)

	Pituitary	Adrenal — Right	Adrenal — Left	Spleen	Thymus	Testes
Pair-fed controls† (60)‡	5.6 .1	10.9 .6	11.6 .4	305.5 27.4	128.2 4.8	851.4 70.3
Choline-deficient (15)	5.5 .2	10.6 .8	11.0 .9	270.8 17.6	131.1 6.0	787.7 103.7
Methionine-deficient (15)	5.8 .1	11.8 .6	12.6 .6	289.7 18.7	178.5 7.4§	387.6 22.3§
Methionine-cystine-deficient (15)	6.0 .2	9.6 .4	10.7 .7	229.3 22.9	175.6 3.6§	176.3 50.6§
Cystine-deficient (15)	5.3 .2	9.5 .4	10.2 .7	265.0 12.3	167.8 3.2§	591.4 97.2

* All weights expressed in mg/100 g body wt. † Pair-fed controls have been grouped due to similarity. ‡ No. of rats. § Indicates P 0.01 for values compared to controls.

Apparent widening of the tunica intima and media with some extension into the tunica adventitio, manifested by cellular vacuolization, is observed in paraffin serial sections of the thoracic aorta in methionine-deficient group (Fig. 3). Except for slight degree of cellular vacuolization, no tunica media hypertrophy occurs in methionine-cystine deficient animals. No increase in plaque formation is observed in any sections analyzed from dietary groups as compared to controls.

Myocardial fibers, cardiac vascularity, endocardium and valve leaflets appear microscopically normal in all groups.

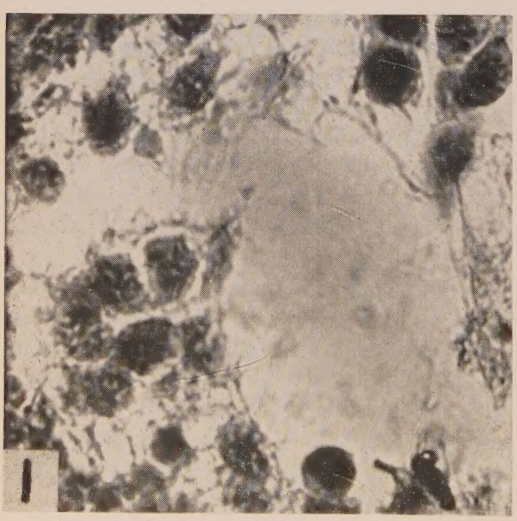

FIG. 1. Sections of rat testes fixed in Helley's fluid and stained with hematoxylin and eosin. High power view (430×) typical of methionine-deficient and methionine-cystine-deficient groups. Seminiferous tubules do not show a compact and orderly arrangement, similar to that of immature animals. Note absence of sperm in tubules.

Sections of liver from choline-deficient animals reveal no abnormalities. Frozen sections of liver stained for neutral fats reveal abnormal accumulations of stainable fat droplets within hepatic parenchymal cells of methionine-deficient animals and to a lesser degree in methionine - cystine - deficient animals. Similar periportal focal fatty metamorphosis (Fig. 3, 4) with moderate to severe nuclear activity and slight hepatocellular necrosis is evident in hematoxylin-eosin stained liver sections of methionine-deficient rats. All sections reveal preservation of lobular architecture. In paraffin liver sections of methionine-cystine-deficient animals, a moderate degree of periportal focal fatty metamorphosis (Fig. 5,6) with evidence of periportal fibroblastic reactions and microscopic areas of mild hepatocellular necrosis is noted. The over-all architectural pattern is well preserved.

Accompanying changes in liver sections are alterations in kidney sections. There is normal kidney structure in the choline-deficient animal. Fig. 7 and 8 reveal that in methionine-deficient rats glomeruli are compactly arranged and vary slightly in size and shape. Bowman's capsule is prominent with slight thickening (Fig. 8). Glomerular congestion and edema are conspicuous. There is variability in capsular spaces. Convoluted and collecting tubules exhibit a cloudy swelling. Although similar microscopic findings are observed for methionine-cystine-deficient animals, the changes are not as marked in this group (Fig. 9, 10). No significant differences from pair-fed controls in the amount of stainable fat are noted in any frozen sections

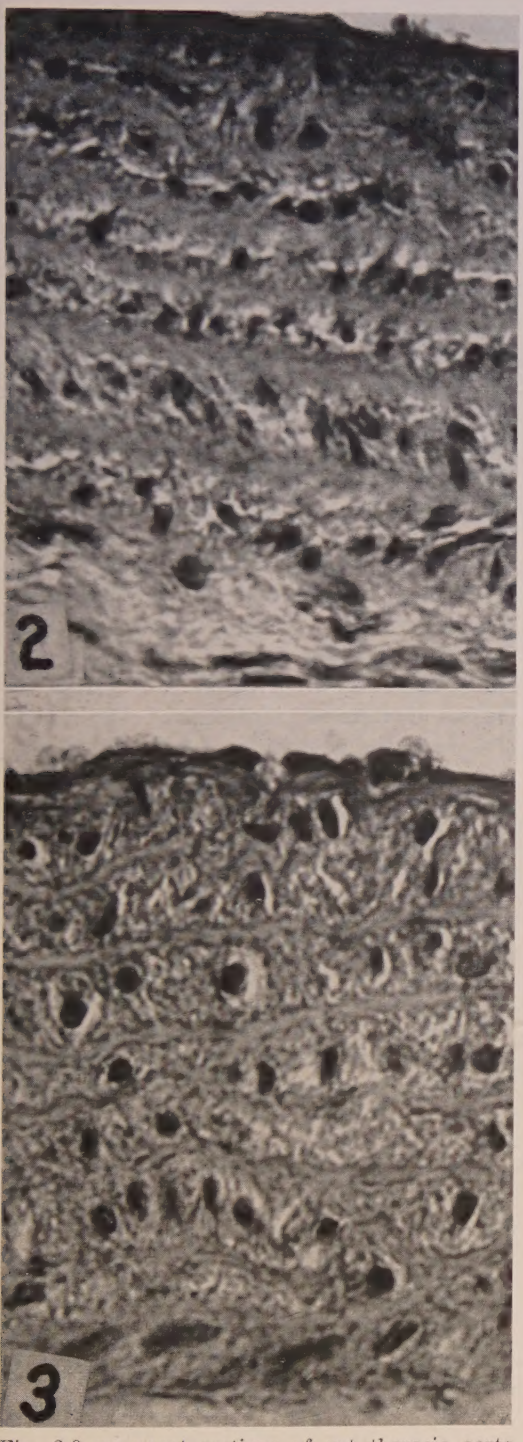

Fig. 2-3 represent sections of rat thoracic aorta fixed in Helley's fluid and stained with hematoxylin and eosin. 430×.

FIG. 2. Typical of pair-fed control, choline-deficient and cystine-deficient animals. Normal architecture.

FIG. 3. Methionine-deficient rat. Widening of tunica intima and media with some extension into tunica adventitio.

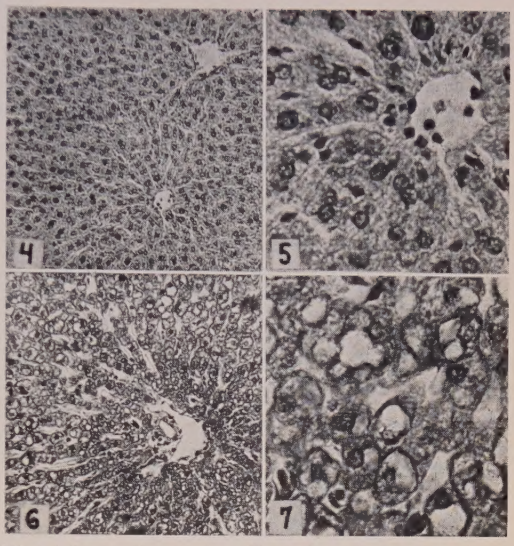

Fig. 4-7. Sections of rat liver fixed in Helley's fluid and stained with hematoxylin and eosin.

FIG. 4-5. Methionine-deficient rat. 50× and 215×. Observe periportal focal fatty metamorphosis.

FIG. 6-7. Methionine-cystine-deficient animal. 50× and 215×. Moderate degree of periportal focal fatty metamorphosis with evidence of periportal fibroblastic reactions is apparent.

of kidney from the deficiency groups.

Routine histologic examination of other organs (thymus, spleen, pituitary and adrenals) reveals no apparent changes.

Discussion. Our results indicate that with a diet containing ample protein and sufficient vitamin and mineral supplement choline is not a critical dietary factor in growth and lipotropism of the male rat subjected to dietary regime of short duration (6 weeks). No evidence was found that exogenous choline-deficiency may be responsible for the pathologic changes noted.

Absence of dietary factors from exogenous sources is often corrected by biosynthesis when necessary basic materials are present for synthesis and conflicting priorities do not exist. Methyl donor function of choline can be provided by methionine or betaine. The lipotropic action of choline, which may be the result of its incorporation into the phospho-

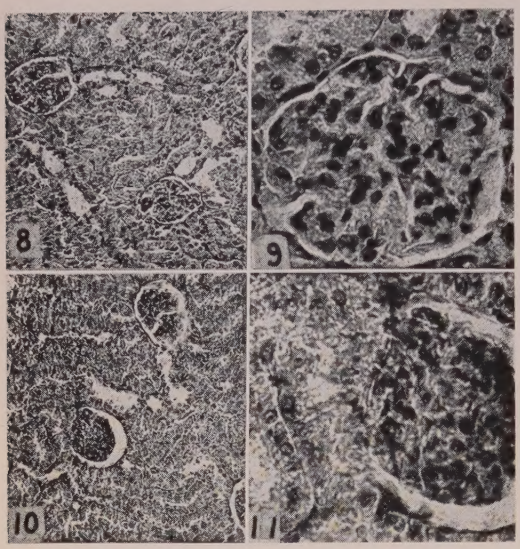

Fig. 8-11. Sections of rat kidney fixed in Helley's fluid and stained with hematoxylin and eosin.

FIG. 8-9. Methionine-deficient rat. 50× and 215×. Glomeruli compactly arranged and vary slightly in size and shape. Glomerular congestion conspicuous. Convoluted and collecting tubules exhibit cloudy swelling.

FIG. 10-11. Methionine-cystine-deficient animal. 50× and 215×. Findings similar to those of methionine-deficient group but not as marked.

lipid molecule, cannot be replaced. However, in the diet there is ample starting material for synthesis of choline and it may be assumed that the lipotropism of the choline-deficient diet under our conditions is the result of endogenous biosynthesis.

The requirements for sulfur amino acids can be satisfied by methionine alone but not by cystine(10). We eliminated methionine from the basal diet by the method of Welch (11) with the result that that methionine and methionine-cystine deficiencies caused development of cardiovascular changes in accord with previous investigations(12,13). Of interest is the finding that methionine and methionine-cystine deficiencies cause increase in thymic weights with accompanying decrease in testes weights. A possible explanation may be found in the work of Martin and Lehr(14) who demonstrated similar thymus-testes inverse weight relation in experimentally induced obstructive nephropathy. They believe that the thymus gland seems to suppress formation or release of excess of certain steroid hormones.

It is extremely interesting that pathologic changes were more extreme in methionine-deficient than in methionine-cystine-deficient animals. The methionine-deficient diets contained an adequate supply of cystine. Cystine added to sulfur-amino acid-deficient diets improves the nutritive value of the diet, increases the demand for methionine for growth and reduces the quantity available for lipotropic activity(15). This appears to be an adequate explanation for our findings.

Summary. 1. Young growing male rats fed 6 weeks a diet containing ample balanced protein and mineral supplement do not require exogenous choline. 2. Similar diets deficient in methionine alone or methionine and cystine cause pathologic changes in testes, kidney, liver and cardiovascular system. 3. Gross and histologic changes are demonstrated in methionine-deficient and methionine-cystine-deficient animals. 4. These changes are more marked in animals fed the diet deficient in methionine alone. Presence of cystine increases methionine requirement for growth and decreases methionine available for lipotropism. 5. An inverse thymus-testes weight relation is noted in animals fed a methionine-deficient diet.

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